A NOTE ON THE COPPER CONTENT OF SEA-URCHIN SEMEN AND SEA WATER

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INTRODUCTION

The addition of copper salts to dense suspensions of unwashed sea-urchin spermatozoa in sea water markedly stimulates the O_2 uptake of the suspension (Rothschild & Tuft, 1950). An investigation of the Cu content of sea-urchin semen and seminal plasma was therefore of interest and is the subject of this note. At the same time the coelomic fluid of *Echinus esculentus* and sea water were analysed for the same metal.

The only recent investigation of the Cu content of sea-urchins was carried out by Webb (1937). He found that the coelomic fluid of *E. esculentus* contained 1·04 μg./ml. Cu, eighty times as much as the sea water (Loch Ine) from which the sea-urchins were taken. The Cu content of this sea water was 0·013 μg./ml., assuming a chlorinity of 19%. According to Sverdrup, Johnson and Fleming (1942), the Cu content of sea water varies from 0·001 to 0·01 μg./ml.

MATERIAL

Semen, seminal plasma (obtained by centrifugation) and filtered or centrifuged coelomic fluid of *E. esculentus*; unfiltered surface sea water collected immediately before analysis in a Pyrex flask off Keppel Pier, Millport.

Before examination, some of the sea-urchins had been kept in the station aquarium. Other sea-urchins were examined immediately after collection.

METHOD

Collection of semen. Semen was obtained as described by Rothschild & Tuft (1950).

Sperm counts. Fuchs-Rosenthal haemocytometer.

Cu analysis. Samples were wet-ashed: dry-ashing in a muffle at a temperature just below red heat resulted in considerable losses of Cu, even though ignition was carried out in the presence of H_2SO_4. The sample was transferred to a 5 ml. flask and heated with several portions of H_2SO_4 (total 1 ml.) to fuming, and then under reflux in the later stages. After thorough digestion, the sample was cleared with 1 ml. of 100 vol. H_2O_2 M.A.R. (Barnes, 1946). After boiling to remove excess peroxide, the clear digest was cooled, transferred to a 10 ml. standard flask and made up to the mark. 2 ml. of 20% (w/v) citric acid was added to prevent interference by iron, followed by 3 ml. ammonia s.g. 880 (ultimate pH 9·2), the solution

again being cooled. After transferring to a small separating funnel, 0·5 ml. of 0·5% (w/v) sodium diethyldithiocarbamate was slowly added, and, after standing 5 min., 1 ml. chloroform; the yellow Cu complex was extracted by shaking for 2 min. After allowing the chloroform layer to settle, it was run off into a 1 cm. micro-Spekker cell and its absorption measured, using violet filters (Ilford 601). A calibration curve was constructed using known amounts of Cu; blanks were run in all experiments.

2 μg Cu/ml. original sample corresponded to a drum-reading difference of about 0·300 divisions. The accuracy of the method is approx. $\pm$0·08 μg. Cu/ml. original sample.

RESULTS

The results of the sea-urchin analyses are shown in Table 1.

Table 1. Cu *content of sea-urchin semen, seminal plasma and coelomic fluid* (E. esculentus)

(All figures for Cu content are averages of two or more replicates.)

Sea-urchin no.	Semen (μg./ml.)	Seminal plasma (μg./ml.)	Coelomic fluid (μg./ml.)	No. of sperm/ml., $\div 10^{10}$
1 (from aquarium)	1·92	2·09	—	1·85
2 (from aquarium)	2·20	0·86	—	2·02
3 (from aquarium)	2·20	—	0·62	1·96
4	1·93	0·88	0·00	1·42
5	1·06	0·47	0·01	—
6	1·01	0·42	0·19	2·76

Three analyses of sea water were carried out. The results, in μg./ml., were 0·0064, 0·0063 and 0·0066, or an average of 0·0064 μg./ml. Table 1 and the sea-water analyses show that Cu is concentrated from sea water to the extent that there is about 200 times as much Cu in semen as in sea water. This, however, applies to semen, less than half of which consists of spermatozoa. Comparison of semen and seminal plasma shows that the spermatozoa concentrate Cu to a greater extent than the figures at first suggest.

The volume of a spermatozoon is about 15μ^3 (Rothschild, 1950). In the case of sea-urchin no. 6, which can be examined as an example, 1 ml. semen, in which there were $2{\cdot}76 \times 10^{10}$ spermatozoa, contained 1·01μg. Cu, while 1 ml. seminal plasma contained 0·42μg. Cu. This means that $2{\cdot}76 \times 10^{10}$ spermatozoa contained 0·76μg. Cu. The concentration of Cu per ml. spermatozoa was therefore about 1·84μg., some 300 times as much as in sea water, or about 3×10^5 atoms of Cu per spermatozoon. In a dense suspension of spermatozoa, containing 10^9 sperm/ml. of sea water, there will be some 6×10^4 atoms of Cu available per spermatozoon, per ml. sea water apart from the Cu they contain and the small amount derived from the seminal plasma.

SUMMARY

1. The copper content of semen, seminal plasma and coelomic fluid of *Echinus esculentus* has been determined. The average figures for sea-urchins examined immediately after collection were: semen, 1·33μg/ml.; seminal plasma, 0·50μg/ml.; coelomic fluid, 0·07μg./ml. The accuracy of the method of estimation was $\pm$0·08μg. Cu/ml. original sample, which means that the amount of Cu in coelomic fluid is not significant. This figure for coelomic fluid is considerably lower than that obtained by Webb (1937) in the only previous examination.

2. The copper content of sea water (surface, off Keppel Pier, Millport) was 0·0064μg./ml.

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STUDIES ON REGIONAL SPECIFICITY WITHIN THE ORGANIZATION CENTRE OF URODELES

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(With Fifteen Text-figures)

INTRODUCTION

It has been known since the work of Spemann (1929, 1931) that there are regional differences in the activities of different parts of the Urodele organization centre in the young gastrula stage. Thus, presumptively, anterior regions, which are at the blastopore lip at an early stage of gastrulation, tend to induce anterior parts of the nervous system, consisting of brain and associated organs, whereas, presumptively, posterior parts tend to induce regions of the trunk or tail. Holtfreter (1936) has mapped more exactly the areas covered by these 'head-organizers' and 'trunk organizers'. Lehmann (1938, 1942, 1945) has identified similar regional differences by observing the effect of temporary exposure of gastrulating embryos to certain deleterious solutions, particularly of lithium salts. He distinguished four zones in the organizer region; the archencephalic zone in the anterior, which later underlies the forebrain, midbrain, eyes, nose, etc.; then the deuteroencephalic zone, in the region of the hindbrain; the trunk zone; and the tail zone, later characterized by the development of the dorsal and ventral tail fins. Dalcq (1947) agrees that there are two different sectors in the brain organizer, which he prefers to call the acrencephalic and chordencephalic instead of archencephalic and deuteroencephalic, as Lehmann does. Nieuwkoop (1947) presents evidence that in secondary induced embryonic axes, the brain is always fully formed up to a certain level, anterior to which it is altogether absent, and on this basis distinguishes at least seven successive zones in the brain.

The evidence for the existence of such specific regions within the organization centre comes, of necessity, from phenomena which imply a mosaic type of development; for instance, inductions in which a part of the organizer causes the appearance of only part of the embryonic axis, or susceptibility experiments in which one region is differentially affected. On the other hand, we have plenty of evidence for considerable lability in the determination of the various regions of the presumptive mesoderm in the early gastrula, for instance in the transplantation experiments of Bautzmann (1932, 1933), Töndury (1937), Raven (1938), the isolations of Holtfreter (1938), and even the original inductions of Spemann (1931), in which the grafts did not always develop into their presumptive fate; while Yamada (1937, 1939, 1940) has shown that even in the neurula the mesoderm is not finally determined. It is therefore necessary to inquire into the status of the regional differences within

the organizer from the early gastrula stage onwards. The indirect evidence would suggest that they must originally be relatively labile and only gradually become more fixed. Direct experiments on the matter are, however, surprisingly lacking up to the present. Hall (1937) found that a posterior organizer, transplanted from an old gastrula into the region of the anterior organizer of a young one, brought about a suppression of head development, the anterior part of the neural system being in the form of a narrow cylindrical tube; while, in the reciprocal experiment, young anterior organizer could be fully incorporated into the posterior of an older gastrula without causing any tendency for brain development in its neighbourhood. This is almost the only series of experiments dealing directly with changes of anterior-posterior regionality within the organization centre of Urodeles, although Bautzmann (1932, 1933) exchanged material between the sides and the midline, and Töndury reversed median strips of tissue which, however, were long enough to bring presumptive ectoderm into the organization centre, while parts of the latter were transferred right outside its boundaries.

As regards the Anura, Waddington (1941) has published the results of reversals of the anterior-posterior axis of a median strip of the organizer, and of exchanges between anterior and posterior organizers of the early gastrula, using the eggs of *Discoglossus*. In general, it was found that the transplanted portions had a strong tendency to carry out autonomously the gastrulation movements proper to their place of origin, and that this prevented their incorporation into the main body of the embryo, on which, in consequence, they showed little sign of exerting a specific regional action. *Discoglossus*, however, is a species in which the morphogenetic movements of gastrulation are particularly vigorous and rapid, involving a very great condensation and elongation of the tissues in the dorsal plane. The evidence to be presented here demonstrates that in *Triton*, in which these movements are more gentle, translocated parts of the organizer can be much more readily controlled by the remainder of the gastrula and thus incorporated into the embryo.

The material to be described here has been accumulated over a number of years, from 1937 onwards. Except for about sixteen organizer reversals, five anterior-in-posterior, and three posterior-in-anterior grafts, which were carried out by Yao, all the operations were made by Waddington, who is also responsible for the detailed examination of the specimens and the writing up of the paper.

All experiments were made on eggs of *T. alpestris*.

EXPERIMENTAL RESULTS

(1) *Organizer reversals*

The eggs were operated at the stage when gastrulation is just beginning, the blastopore being represented by a fairly broad, shallow groove, lined by flask cells, but with as yet no definite archenteron extending inwards from the surface. The operation consisted in excising a rectangular region, bounded by the blastopore groove on the vegetative side, and extending animal-wards about to the boundary of the presumptive mesoderm; its width from side to side was rather greater than the

extent of the blastopore (see Fig. 1). In freeing this flap of tissue from the embryo it is necessary to dissect it away from the underlying cells in the immediate neighbourhood of the blastopore. In doing so, a considerable quantity of the deeper-lying presumptive prechordal mesoderm was left attached to the fragment, but the boundaries of this tissue are not clear in the living egg, and some of it was probably left *in situ*. After the fragment had been completely separated from the egg, it was rotated about an axis perpendicular to the egg surface, and replaced, so that its original animal side lay next to the blastopore, while the original blastopore lip lay near the boundary of the presumptive mesoderm.

Some thirty embryos operated in this way are available for examination; most of them were fixed in late tailbud stages. Of these, nineteen appear to be completely normal in every way. In them regulatory processes originating from the main body of the embryo must have completely overcome the initial disorganization caused by the reversal of the median strip of the organizer.

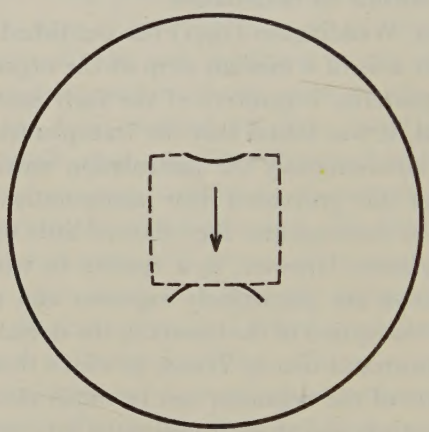

Fig. 1. Diagram of operation, reversal of organizer.

In another group of four embryos, there is some disturbance of the notochord. Usually this takes the form of an enlargement of this organ in the anterior region or a reduction of it in the posterior, or both these effects may occur together. This is probably due to a tendency for the displaced blastopore lip to begin invaginating in its new, more anterior position; if material invaginating in this position were then joined by other tissues proceeding from the blastopore lip in the usual way and carried forward with them, one might expect to find an excess of material in the anterior part of the archenteron roof, while if the remainder of the embryo failed to compensate completely to such a process, a deficiency in more posterior regions would result.

In another small group of four or five embryos (partly overlapping with the last group) there is greater or lesser degree of spina bifida posterior. In these embryos it is clear that the reversed section has acted as an impediment to the normal gastrulation movements of the remainder of the egg, so that the latter have become split into two streams, each of which has given rise to a half embryo. In most cases, it is probable that the impediment has occured because the displaced blastopore material

has attempted to invaginate in its normal direction (which, in its reversed position, would be towards the blastopore) but has been prevented in its movement by the invagination proceeding in the unaltered lateral parts of the germ, and has thereby been reduced to immobility. In similar experiments on the Anuran *Discoglossus* (Waddington, 1941) such an autonomous invagination of the reversed piece usually succeeds very much more completely, possibly because the gastrulation movements in that form are much more vigorous and rapid than they are in *Triton*. Only one case of a partially successful autonomous invagination of the reversed section has been found in the latter. In it (embryo no. OR 57, Fig. 2) the reversed graft formed

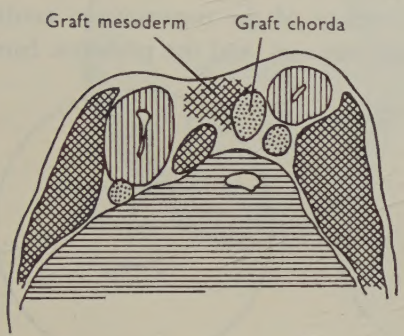

Fig. 2. No. OR 57. Section through mid-trunk region. The independent invagination of the graft has split the host axis into two halves, each of which has undergone considerable regulation.*

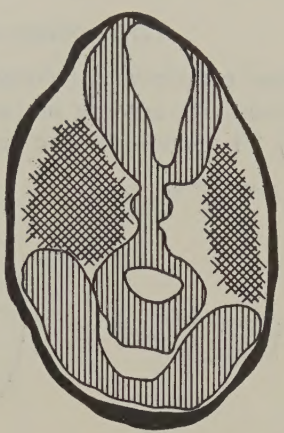

Fig. 3. No. K 6*b*–3. Organizer reversal. Section through eye region, exhibiting microcephaly.

a knob-like outgrowth which was originally seen protruding from the centre of the neural plate. In the later stages it was visible, caught between the two sides of the neural groove, which had folded together pinching it between them. In sections, this protuberance is found to contain a number of axial tissues, including chorda, somitic mesoderm and probably neural tissue, which, as is well known (Holtfreter, 1938) can be developed by isolated fragments of the presumptive mesoderm. The presence of this mass of extraneous tissue causes the main neural system of the embryo to have the appearance of a spina bifida; and it is noteworthy that in this case each of the halves of the neural tube has to a considerable extent become regulated into a bilaterally symmetrical cross-section, so that we have almost a complete doubling of the axis. In other spina bifidas described in this paper, the two half-axes are usually widely separated by an expanse of uncovered endoderm, from which the graft tissues have fallen away; and in these cases there is little tendency for the regulative restoration of their bilateral structure. Regulation leading to complete doubling of the axis has, however, been described by Bautzmann (1932) in

* In all the diagrammatic drawings of sections, the neural tissue is lined vertically, the endoderm lined horizontally, the mesoderm cross-hatched and the notochord dotted.

other experiments, in which the invagination stream was divided into two without complete loss of tissue continuity between the two sides. A similar phenomenon has been demonstrated in the chick embryo (Abercrombie, 1950; Abercrombie & Morgan, 1950).

Finally, some three or four embryos show a slight under-development of the head (Fig. 3). This microcephaly is almost certainly due, not to a direct action of the reversed section, but to a partial inhibition of invagination, leading to deficiency in the anterior archenteron roof, caused by the antagonistic action of the graft on the normal movements of the remainder.

(2) *Exchanges of anterior and posterior organizer*

In these experiments, rectangular flaps of tissue were excised, containing either the presumptive anterior or posterior median regions of the organization centre (Fig. 4). The anterior region being removed from one egg, and the posterior from

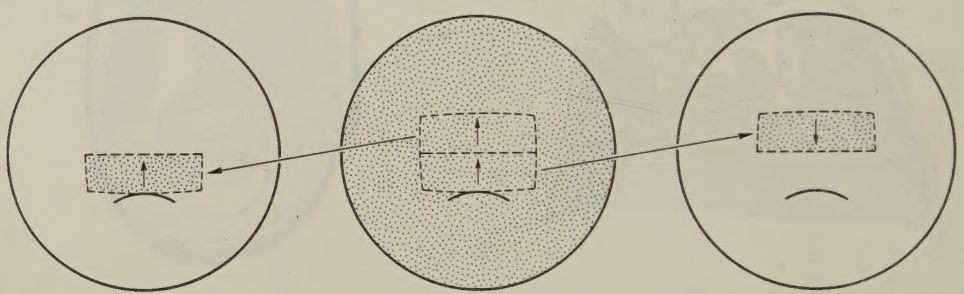

Fig. 4. Diagram of operation, exchanges of anterior and posterior organizers. Centre: donor; on left: post. in ant. *aa*; on right: ant. in post. *ap*.

another, the two pieces were exchanged, so that one embryo received a posterior-in-anterior graft and the other an anterior-in-posterior. The grafts were made either in their original orientation, i.e. with the originally anterior material still towards the anterior (a so-called *aa* graft) or with the anterior-posterior axis reversed (an *ap* graft). Some of the donor embryos were vitally stained with Nile blue, so that the graft could be recognized among the host tissues.

(*a*) *Posterior-in-anterior grafts.* There are thirty-eight specimens in which this operation was performed on the young gastrula in which the blastopore is still an open groove. In nine of these the anterior-posterior axis was reversed.

Ten of these embryos appear perfectly normal in every way. Regulation can, therefore, be quite complete in favourable cases.

The remaining embryos show various types of abnormality. The commonest of these are: microcephaly, which in extreme cases takes the form of cyclopia, or even the total absence of eyes; and spina bifida. The explanation of the latter condition is fairly obvious; the relatively inactive posterior region has failed to invaginate, or has invaginated less rapidly than the lateral parts of the presumptive mesoderm, and has therefore acted as a block, splitting the archenteron roof in two. In the weaker grades of this, one finds a doubling of the chorda, and perhaps some doubleness

in the neural tube. There are also cases in which the only abnormality is a slight enlargement of the notochord, usually in the mid-trunk region (Fig. 5). These are probably to be attributed to a certain regulatory production of extra chorda by each lateral part of the host, during an early stage at which the graft is still unassimilated, followed by fusion between these host chorda masses and the chorda derived from the graft itself; they can therefore be regarded as similar in nature to the spina bifidas, which differ in that the later fusion fails to take place.

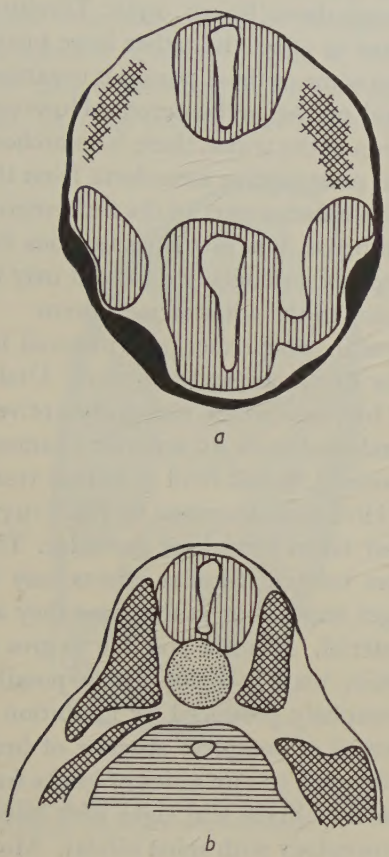

Fig. 5. No. E106*b*–7. Post. in ant. *aa*. Normal embryo, except for enlargement of chorda in trunk region, shown in *b*.

Fig. 6. No. K 8*b*–2. Post. in ant. *aa*. Spina bifida.

In higher grades of spina bifida, the embryo is completely split up the back. Usually, in such cases, the graft tissue finally separates itself completely from its host, drops off and is lost. The mid-dorsal region is then occupied by a broad expanse of naked endoderm, on each side of which runs a half-axis, which often curls up dorsally as a bifid 'tail' (see Fig. 6). There is usually little tendency for these separate half-axes to regulate to a bilaterally symmetrical cross-section.

The microcephaly can be brought about in a number of different ways. The least specific of these is by a general inhibition of gastrulation movements, probably

leading to a deficient development of archenteron roof in the anterior. In experiments in which the graft was vitally stained, it could in a number of cases be seen that the grafted posterior organizer had not moved forward far enough to underlie the head, as it should have done if it had behaved entirely in accordance with the position in which it had been placed. Presumably this material, which had not yet reached the stage of invagination at the time it was removed from the donor, is at first relatively passive; we know that the surroundings of the blastopore lip can induce a tendency to invagination in indifferent material placed among them (Raven, 1938; Töndury, 1937); but this would undoubtedly take some time to affect the rather large pieces involved here, and it is not unexpected to find that some of these posterior organizer grafts lag behind and fail to reach the front end of the archenteron. However, even when the graft only reaches the anterior region of the trunk, there is an archenteron roof farther forward than this, formed from presumptive mesoderm from the main body of the embryo. The relative size of this anterior part of the archenteron roof has not been directly measured in such embryos, but in such situations the brain may be reduced in size, leading to microcephaly, cyclopia, etc., and it may be presumed that this is a consequence of a deficiency in the anterior mesoderm.

Reduction of the head also occurs in embryos in which the graft material invaginated more rapidly and reached the anterior of the archenteron roof. Under these circumstances, the brain will be underlain by tissue which was presumptively posterior organizer; and unless this tissue is endowed with an anterior character by regulatory processes within the sheet of mesoderm, it will tend to induce trunk rather than brain structures. Phenomena of this kind were described by Hall (1937), when he grafted fragments of posterior organizer taken from later gastrulae. This can be confirmed from the present material (see below). Similar effects may be produced when the grafts are taken from younger stages, but in that case they are much less frequent than they were in Hall's material. It is not possible to give an exact figure for the frequency of their occurrence, since it is not always possible to distinguish them clearly from cases of microcephaly produced by inhibition of gastrulation; but whereas Hall found twenty cases of complete absence of brain and only four in which the brain was partly developed, in our series of grafts from the early gastrula there are ten completely normal embryos and eight with microcephaly (apart from several which combine microcephaly with spina bifida). Moreover, in the present series, the microcephaly is much less extreme than that described by Hall as typical. Whereas in his cases the brain was reduced to a simple tube with narrow lumen, in ours the microcephaly usually amounts to no more than a reduction in size, an under-development or complete failure of the optic lobes, and a smoothing out of the divisions between the lobes of the brain (Fig. 7). The most extreme cases of microcephaly found in the young gastrula grafts have been in embryos in which the anterior-posterior axis of the graft was reversed (*ap* grafts), several of which have led to a condition approaching, though not attaining, that described by Hall.

One can conclude that the presumptive posterior region of the organizer at the early gastrula stage can be completely converted, in these grafts, so as to develop into anterior tissues and so as to induce the anterior parts of the neural system. This

conversion is perhaps less easy when the graft is made with a reversed orientation, as is evidenced by the more extreme microcephaly which may be produced in such cases, but even among them some instances of perfect regulation occur.

The difference of behaviour of these early grafts and those in which the posterior organizer is taken from embryos in later gastrula stages has been directly confirmed in a special series of operations from which sixteen specimens are available. The dorsal blastopore lips, including both the superficial and the already invaginated layers, were removed from mid-gastrulae with smallish yolk plugs and grafted into the anterior organizer region of young gastrulae, in the manner described by Hall. The donors were somewhat younger than Hall's, being nearer Harrison's stage 12 than 13. The results were more or less intermediate between those in the earlier

Fig. 7. No. E 107*b*–3. Post. in ant. *ap*. Section through anterior of mid-tailbud stage, exhibiting moderate microcephaly.

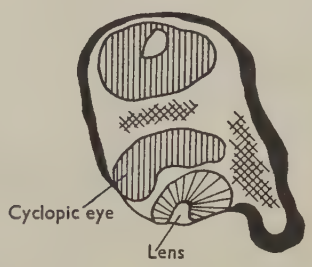

Fig. 8. No. H 19–2. Post. of small yolk-plug gastrula in ant. of young gastrula. Section of anterior end of head, late tailbud stage, showing extreme microcephaly and cyclopia.

grafts described above and those recorded by Hall. No perfect embryos were obtained when the graft came from an embryo later than the horse-shoe blastopore stage. In all the others, the head was considerably reduced, with either two very small eyes, or, more usually, one small cyclopic eye (Fig. 8). The formation of extra notochord in more posterior regions was also common, but complete spina bifida was rather rarely found, probably because in these grafts the posterior organizer was already in process of invagination and could therefore more easily keep pace with the movements of the host.

(*b*) *Anterior-in-posterior grafts.* Thirty-four embryos are available, from experiments in which an anterior organizer was placed in the position of the posterior organizer of a young gastrula. Of these thirteen are apparently quite perfect, two

of them from the small group of seven in which the anterior-posterior axis had been reversed.

The others show, to a greater or less extent, the same type of abnormalities due to disturbances of gastrulation which have been described above, namely enlargement or doubling of the notochord, spina bifida and microcephaly. In several cases the spina bifida is quite slight, as in Fig. 9. The microcephaly is also usually not

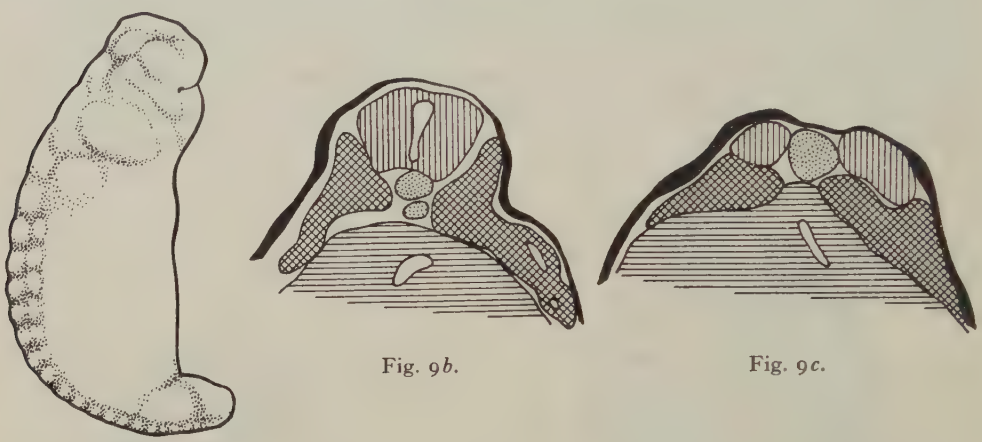

Fig. 9*a*. Fig. 9*b*. Fig. 9*c*.

Fig. 9. No. K 8*b*–4. Ant. in post. *aa*. *a*, tailbud stage, with slight microcephaly; *b*, double notochord in neck region; *c*, spina bifida in trunk.

Fig. 10. No. 107*b*–13. Ant. in post. *ap*. Enlarged notochord in posterior trunk region. Under the notochord is a mass of neural tissue derived from the graft, which has been carried forward with the invaginating host mesoderm.

pronounced. It must certainly be attributed to a partial inhibition of gastrulation, leading to a reduction in the mass of the anterior archenteron roof. In the *aa* grafts the mechanism of this reduction is rather obscure; it may be simply a consequence of the damage done during the operation, and the difficulty of obtaining satisfactory healing of the rapidly elongating anterior organizer into a region which is itself undergoing active gastrulation movements. In the *ap* grafts, it is easy to see that there would be a tendency for an invagination stream to proceed from the graft in a direction opposite to that of the main mass of mesoderm moving forward from the host blastopore. In *Discoglossus* these two streams usually remain quite distinct and come into direct conflict, when the graft stream may be bodily bent round and

carried forward embedded in the host mesoderm (Waddington, 1941). Only one example of this has been found in the *Triton* material, but there are several other cases in the *ap* series in which the chorda is greatly enlarged in the posterior trunk region (Fig. 10), and reduced or altogether absent in the anterior, which also exhibits microcephaly, and these can also be accounted for by a partially successful attempt of the anterior graft to invaginate according to its presumptive fate.

A few specimens are available (five in sections, a similar number observed *in toto* but not sectioned) in which the anterior organizer of a young gastrula was substituted for the posterior organizer of an older host, in Harrison's stage 12. Hall has shown that such grafts may be perfectly assimilated in even later stages, and perfect assimilation was, as might be expected, also found in many cases here. However, in *ap* grafts of reversed orientation regulation was not always perfect, and extra masses of chorda could be found in the posterior trunk region.

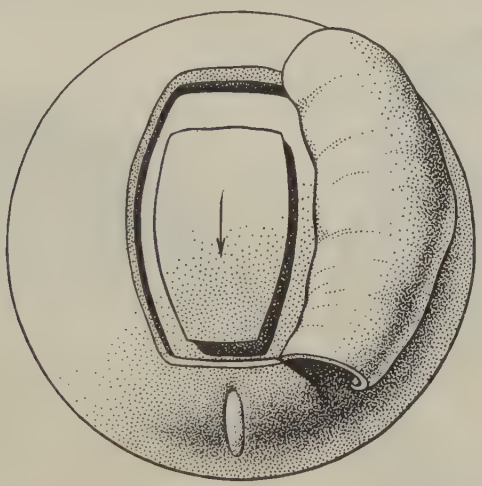

Fig. 11. Diagram of operation. Extra archenteron roof added, in reversed orientation, under the presumptive neural plate of a late gastrula. The anterior-posterior extent of the added material was actually greater than shown in the diagram.

(3) *Addition of extra archenteron roof*

We have seen that regulation may be perfect in the early gastrula stage; Hall has shown that the posterior organizer of a late gastrula has a regional character which cannot be overcome by the presumptively anterior surroundings of a young gastrula; and one wonders whether this character is so firmly fixed that it cannot be altered by influences present in the late gastrula. A small series of experiments was made to test this. In a late gastrula in which the yolk plug had just become slit-like (Harrison's stage 13 or slightly later), the whole presumptive neural plate was freed along one side and round the anterior, and a second archenteron roof, removed from another similar gastrula, placed with reversed orientation on top of the normal roof, the neural ectoderm then being carefully folded back and allowed to heal over the double layer of mesoderm (Fig. 11). It is impossible to carry out this operation as perfectly

as the description would suggest; there is a strong tendency for the second roof to roll up, and some damage is usually done to it while spreading it out flat over the roof of the host, and although the reversal of its anterior-posterior axis presents no difficulty, it is difficult to recognize with certainty its midline and still more difficult to be sure that after the ectodermal covering is restored this midline is accurately above that of the host. In some cases, in fact, the whole added archenteron roof has shifted to one side of the host axis, and has there produced an induction of normal type.

Only about seven embryos are available in which the operation was tolerably successful. This is not nearly enough to elucidate all the consequences which may follow such an addition to the mesoderm layer. There are, however, three instances in which a relatively perfect and complete embryo has been formed; embryos, that is to say, which differ from normal only in the exaggerated thickness of their axial mesoderm (somites and chorda) (Fig. 12). And another embryo is fairly normal

Fig. 12*a*.

Fig. 12*b*.

Fig. 12. No. E 128*b*–2. Extra archenteron roof added, *ap*. *a* shows a relatively normal head; *b*, the trunk region with unusually thick mesoderm.

except that one side of the neural system is over-developed and larger than the other. These suffice to demonstrate that regulation of anterior-posterior quality is still possible in the late gastrula stage, provided suitable influences can be brought to bear.

In all the other three abnormal embryos the notochord is lacking in large sections of the trunk, its place being occupied by a thick layer of apparently somitic mesoderm (Fig. 13); in two of them the head is very badly developed, with no proper brain or eyes. A much larger series would be necessary to reach a full understanding of the various abnormalities, but it may be surmised that in these cases the midline of the added archenteron roof did not coincide with that of the host, so that axial mesoderm

was brought into contact with more lateral material, and altered in its development (cf. Yamada, 1937–40).

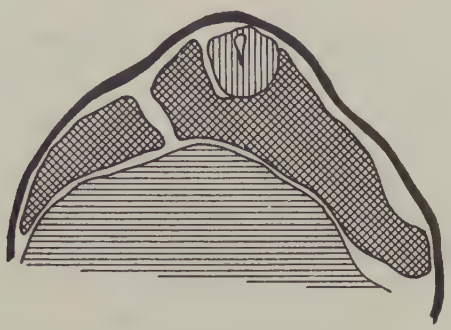

Fig. 13. No. E. 89*b*–3. Extra archenteron roof added, *ap*. Section through trunk region, showing absence of notochord and thick somitic mesoderm.

DISCUSSION

The evidence presented above makes it clear that a fully normal embryo may be formed after the reversal of the organizer, or the exchange of its anterior and posterior parts, when the operations are made in early gastrula. Such a result involves the complete regulation of the topographically altered organization centre. As Raven (1948) points out, 'regulation' is an 'unclear and heavily charged word', which it is in many ways desirable to supersede by more precise expressions. According to his recommendations, one should classify the phenomena in question (in terms originally due to Dalcq) as a 'teleogenetic paragenesis', i.e. the production of a normal end-result by means of an abnormal process of development. Another way of describing the same situation is to use a term originally introduced by Waddington & Schmidt (1933), and say that the individuation field of the primary organizer can restore its normal configuration after being disturbed by the reversals or exchanges in question.

It was pointed out in the Introduction that this differs sharply from the result of similar experiments in the Anuran *Discoglossus* (Waddington, 1941), and it was hinted that a cause of this difference may be sought in the different tempo and extent of the gastrulation movements in the two species. At present this seems to remain the most plausible suggestion. Certainly the inherent tendencies to perform specific types of movement, which were the most interesting and as yet inexplicable feature of the *Discoglossus* experiments, are very much less marked in *Triton*. They are, however, expressed in certain ways, for instance, after organizer reversals; but in general the power of inducing appropriate movements, which the main bulk of the organization centre is known to possess (Raven, 1938; T'öndury, 1937) usually succeeds in overcoming the autonomous tendencies of the graft. The nature of the inductive influence which has such effects on tissue movements is one of the most fascinating and obscure problems of physico-chemical embryology.

In the chick embryo, the possibilities of regulation seem rather similar to those in *Triton*, since Abercrombie (1950) has demonstrated complete regulation following

the reversal of various median portions of the organization centre, comprising parts of the primitive streak.

There is evidence in the present experiments for an increasing fixity of regional character during the progress of gastrulation in *Triton*. Thus when a young posterior organizer is placed in the anterior region of an early gastrula, a normal embryo may be formed; but when, in an exactly similar experiment, the graft is from an older stage, it cannot be fully converted, and some degree of microcephaly results, which reaches an extreme form when the graft is from the still older stage used by Hall. Nevertheless, the regional character is even then by no means irrevocable, as is shown by the formation of normal embryos following the addition of an extra archenteron roof, in reversed orientation, to a late gastrula of about the stage Hall employed. There are, perhaps, two main respects in which this experiment differs from the earlier one. In the first place, the host embryo is here an old gastrula, whereas in Hall's experiments the posterior graft was made into a young stage. At this greater age the regional structure within the host would be more intensely developed, and may be able to exert a stronger influence on the graft. Secondly, the added archenteron was placed inside the gastrula, lying with its whole surface against that of the normal archenteron, and it seems reasonable to suppose that in this situation it is more accessible to influences from the host than it would be when only attached to the sheet of host mesoderm along its edges, and with the necessity to undergo the movements of gastrulation. In any case, a lability of regional determination at this stage cannot be considered surprising in view of Yamada's demonstration that even later, in the neurula, the histogenetic fate of the various types of mesoderm is not finally fixed.

Considerable discussion has taken place in recent years as to the nature of the regional differences within the organization centre. The main debate has been between those, like Dalcq & Pasteels (1937, and later), who argue that these are primarily quantitative, and others, of whom Lehmann (1938, and later) is one of the most prominent, who urge the importance of qualitative differences. It is essential, of course, to specify precisely the stage of development to which one is referring. It is certain that there must eventually be qualitative differences in the chemical constitution of the different parts of the axis; the question is at what stage they arise, and whether they are preceded by a field of quantitative differences.

As regards the situation during the phase of gastrulation, before the appearance of the neural plate, there seems now general agreement as to the facts which have to be explained. It is conceded by both sides that chordal part of the archenteron roof behaves in most ways as a single unified region, while the pre-chordal plate acts as though it were to some extent independent. Dalcq (1947), in consequence, supports the view that 'quantitative relations are fundamental', but makes a 'reservation for the relative specificity of the pre-chordal region', and considerably more hesitantly for the parachordal mesoderm. Lehmann (1945, p. 318) emphasizes the special position of the pharyngo-dorsal blastema (which corresponds to Dalcq's pre-chordal plate), but admits that there is a good deal of evidence for mainly quantitative differences within the chorda-mesodermal region.

Both these alternatives seem to be somewhat too simply posed. There are other possibilities to be considered. Five of them were already listed at an early stage in the discussion (Waddington, 1937).

(1) Different regions of the gastrular organizer might contain different evocator substances, each specific for a particular region of the axis. The evidence, from extraction experiments and studies on regulation, is all against this hypothesis.

(2) There may be a basic evocator substance, which, in different regions, is 'modulated' by other additional substances or slight changes in its structure (for instance, if the basic evocator is a protein). It can now be taken as almost certain that there is a basic evocator; and the eventual development of specific local chemical differences in stages later than the gastrula could be considered modulations of it; but whether such modulation has already occurred during gastrulation is still open for discussion.

(3) The regional differences could be quantitative, particular regions being induced by particular absolute concentrations of evocator. This would seem somewhat unlikely, both on account of the regulative phenomena encountered, and because it would lead one to expect more evidence of regional specificity in experiments with dead organizers, evocator extracts, etc., than is actually found.

(4) The regional quality could depend on relative concentrations, i.e. on quantitative gradients. This would be the simplest explanation of regulation, but it would be difficult on such a basis to account for those cases where regional specificity has been shown by abnormal evocators, as in Chuang's (1938 and 1940) and Toivonen's (1938) experiments.

(5) The possibility was suggested (Waddington, 1937) that specificity might depend on some 'specific spatial properties', those mentioned as examples being related to the micro-structure, such as the formation of partially oriented aggregates, resulting in liquid crystals, specific surface properties, etc. According to the fashion of the day, hypotheses such as this tend, perhaps, to be rather too easily accepted in the capacity of *deus ex machina*, and it seems advisable to hold such modes of explanation in reserve until there is some positive evidence in favour of them, or at least till all more readily verifiable alternatives are exhausted.

No one of these five possibilities (including the extreme qualitative hypothesis, no. 1, and the extreme quantitative ones, nos. 3 and 4) seems fully satisfactory. It is more probable that the actual situation corresponds to some combination of them. The existence of regionally specific inductors in dead adult tissues, demonstrated by Chuang and Toivonen, and the hints that similar effects can be produced by dead material from later embryos (see Lopashov, 1936; Waddington, 1937) strongly suggest that something comparable to the postulated modulation of the basic evocator does occur after the stage of gastrulation; while the ease of regulation at the earlier stage, new evidence of which has been brought forward in this paper, is most easily accounted for in terms of a quantitative hypothesis such as no. 4 above. Moreover, if attention is paid to the actual events which are occurring while the evocator is being produced, it is easy to see ways in which it may plausibly be supposed that such quantitative differences arise. Indeed, the phenomena of gastru-

lation invite our attention to two further types of factor, one concerning the time relations of the various processes, and the other to spatial considerations, not on the scale of 'intimate structure' originally contemplated in hypothesis no. 5, but on the more straightforward scale of the gastrulation movements themselves.

Attention has earlier been drawn (Waddington, 1940) to some of the phenomena which would almost inevitably follow from the mere physical events of gastrulation. The neural evocator is liberated at the blastopore, in the stream of mesoderm which moves forward as the archenteron roof. From this it diffuses into the overlying tissue, and also presumably laterally from the midline towards the lateral mesoderm. One can probably also presume that the liberation is not a sudden

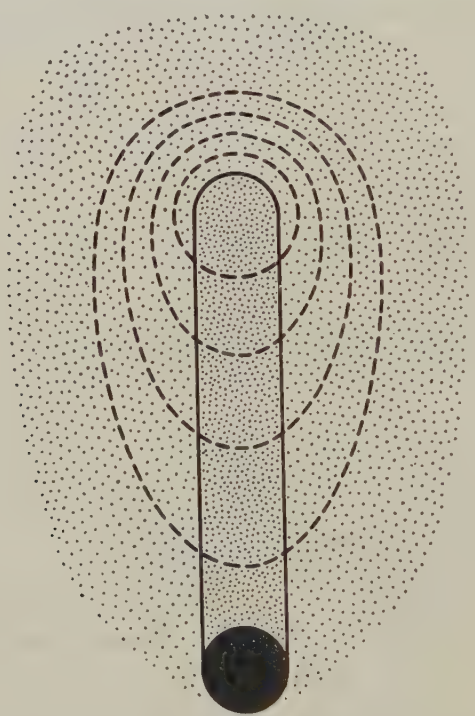

Fig. 14. Diagram showing the yolkplug blastopore (black) from which there extends forwards the layer of mesoderm (dotted). The evocator may be supposed to be liberated in the archenteron roof (close dots), and then is distributed in accordance with the concentration contours shown.

process, complete in the tissue immediately after it has invaginated, but that it continues for some time during the forward movement of the mesoderm sheet. A process of this kind would give rise to some sort of pear-shaped distribution of evocator within the mesoderm (Fig. 14), but we know too little about the exact directions of movement within the mesoderm, the initial concentrations of precursor and rates of liberation, diffusion, etc., for it to be worthwhile attempting to calculate a more precise shape. The pear-shaped region would, however, increase in area as it moved forward, and probably the concentration in the centre of it would increase. Nieuwkoop (1947) has drawn a figure of a rather similar 'mesodermal gradient',

but instead of deriving it from considerations of the production and diffusion of the evocator, he merely postulated that it already exists in the presumptive mesoderm of the early gastrula, waiting to be passively folded in to form the archenteron roof.

Nieuwkoop suggests that this mesodermal gradient system reacts with an ectoderm which is also characterized by a field of quantitative gradients. There is, however, no independent evidence for the existence of such a system; in fact, what evidence there is (e.g. Holtfreter, 1933*a*) is against it, and Nieuwkoop can do no better than argue that the experimental analyses made up to the present have been too crude to reveal it. There are, however, two other major factors which must be involved and which may have an influence; and a consideration of these suggests that all the

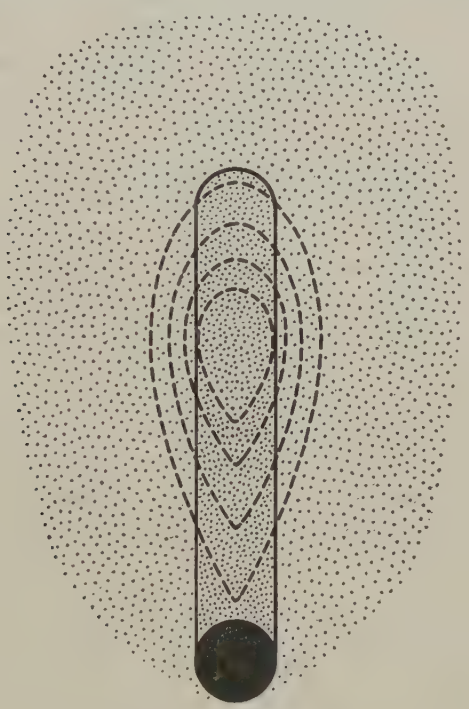

Fig. 15. Concentration contours in the ectoderm, which moves down over the mesoderm towards the blastopore.

known facts may be explained without calling on such an insecurely founded entity as an ectodermal field.

The first of these factors is that of time. During the period when the evocator must be supposed to be diffusing into the presumptive neural plate, the latter is moving towards the blastopore, passing over the sheet of mesoderm which is flowing the opposite way. Again we know too little of the details to make it worth attempting an exact calculation of the consequences of this, but it is easy to see in general terms that the concentration contours in the ectoderm will differ from those in the mesoderm somewhat in the manner indicated in Fig. 15. Moreover, there is a

second variable to be considered: these concentrations will have been accumulated over different periods of time; the most anterior region of the neural plate will have been underlain by evocating mesoderm for a shorter time than some of the more lateral and posterior tissue which may lie within the same concentration contour.

Some evidence for the existence of such concentration gradients is already available. In the uninvaginated mesoderm, Bautzmann (1926) showed that the organization centre was relatively sharply bounded as against the more lateral mesoderm which showed no inducing capacity; in the neurula, on the other hand, Holtfreter (1933*b*) found that lateral plate was almost as powerful an inductor as axial mesoderm. It is, however, the stage between these two, that of gastrulation, in which we are interested, and at that stage the existence of an inductive capacity in the lateral plate, definite but weaker than that of the archenteron roof, has been demonstrated by Waddington (1936). More recently, Raven & Kloos (1945) have presented evidence for a similar lateral gradient within the axial mesoderm. Gallera (1947) and Damas (1947) (see also Dalcq, 1947) have also found a cranio-caudal decrease in the 'differentiation potential' of the ectoderm of the neural plate. As yet, comparatively little is known about such gradients. What information we have suggests that they are similar, in that in both of them regions at the 'high' end induce or develop into well-formed neural organs while the 'low' end forms only neural crest. This suggests that the medio-lateral gradient, which would involve only concentration, is effectively similar to the antero-posterior one, which would also involve the length of time over which the concentration had been accumulated. But further work may show that this time factor cannot be left out of account. In particular, the distinction between the pre-chordal plate and the remainder of the archenteron roof may prove to correspond with the difference between the gradient in front of the region of highest concentration, in which the evocator content has been rapidly built up, and that in the more posterior and lateral regions in which it has been slowly accumulated.

Thus the conditions for the production and diffusion of an evocator within the dynamic topographical situation of the gastrula are sufficient to suggest an extremely flexible hypothesis which, by suitable assumptions as to the relative rates of the various component processes, could be made to fit many different facts; but it is worth noting that there is no reason why these assumptions should not be open to independent testing.

There is, however, one class of facts which cannot be explained by the considerations which have so far been advanced. We have seen in the experimental part of this paper, in confirmation of Hall's earlier results, that the posterior organizer of the mid-gastrula is already different to the anterior organizer; but at this time it still lies on the surface of the embryo; it cannot therefore differ merely in a lower content of free evocator, since according to the views so far advanced no evocator would have yet been produced within it. Moreover, when it is transplanted to the blastopore lip of a young gastrula and from there invaginates so as to reach the anterior end of the archenteron roof, we have as yet no way of understanding why it does not develop a full quota of evocator and thus act as an anterior organizer. There is,

however, another factor which might come into play here. The posterior regions of the organizer, during their movement towards the blastopore, are themselves for some time underlain by active inducing material. It is conceivable that this preliminary submission to the diffusing evocator, as well as the evocator liberation after invagination, is connected with the determination of regional properties.

A careful consideration of the actual events of gastrulation seems therefore to force on our attention a number of variables in the spatio-temporal conditions of evocator production and diffusion which might go far to explain the facts about regional determination at that stage as far as they are yet known. These factors urgently demand investigation. The same is true of the closely allied question of why it is that a piece of ectoderm grafted so as to remain on the surface above archenteron roof becomes neuralized, whereas a similar piece which passes below the surface into the archenteron roof becomes mesoderm.

SUMMARY

1. In young gastrulae of *Triton alpestris* the median part of the organization centre immediately in front of the blastopore was excised and replaced after reversal of its anterior-posterior axis. Completely normal embryos developed in many cases, but in others there was some degree of microcephaly or spina bifida.

2. Similar normal embryos can develop after exchange of the anterior and posterior regions of the organizer, either with normal or reversed orientation.

3. The spina bifida which frequently appears is a consequence of the impediment offered by the graft to the normal gastrulation movements.

4. Microcephaly may also result if the presence of the graft prevents the proper development of the anterior part of the archenteron roof.

5. It may also be caused when the posterior organizer region is brought into the anterior region, if the host fails to convert it into anterior material. This occurs the more frequently the older the grafted posterior material is.

6. Even at the end of gastrulation (slit yolk-plug stage) the regional character of the archenteron roof is not finally determined. A fairly normal embryo (with overthick mesoderm) may develop if an extra archenteron roof is added with reversed orientation between the normal archenteron roof and the presumptive neural plate.

7. The determination of the regional structure of the archenteron roof and the neural plate is discussed. Attention is drawn to the spatio-temporal factors involved in the production and diffusion of the evocator with the dynamic system of the gastrulating egg.

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OSMOTIC REGULATION IN MOSQUITO LARVAE

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(With Plate 2 and Two Text-figures)

I. INTRODUCTION

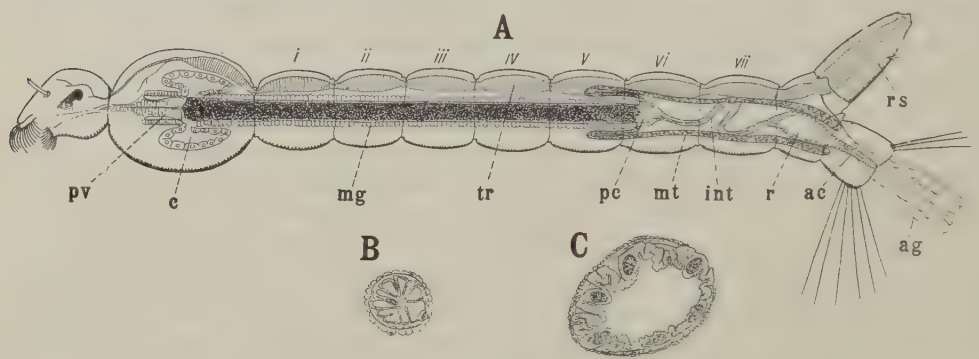
The ability of various mosquito larvae to regulate the osmotic pressure of the haemolymph in the face of changes in the external medium has been clearly established by the work of Wigglesworth (1938) and of Beadle (1939). Particular interest attaches to two species of the genus *Aedes*: *A. aegypti* L., which is a fresh-water form incapable of living in sea water, and *A. detritus* Edw., which is normally found in saline, often in highly saline, waters.

Wigglesworth has shown that in *A. aegypti* the osmotic pressure of the haemolymph is equivalent to 0·80–0·89% NaCl with an average chloride content equivalent to 0·30% NaCl. More than half of the total osmotic pressure is thus exerted by non-chloride, presumably organic, solutes. When the larvae are placed in artificial sea water of various dilutions it is found that both the osmotic pressure and the chloride content of the haemolymph remain constant until the concentration of the external medium reaches 0·65–0·75% NaCl. In more concentrated media the chloride content of the haemolymph increases and the total osmotic pressure is thereby maintained slightly in excess of that of the medium up to an external concentration of 1·6% NaCl, at which the larvae die rapidly. In earlier papers Wigglesworth (1933*a-c*) showed that whereas the general body surface was relatively impermeable to water and salts, these substances penetrated readily through the anal gills; and Koch (1938) demonstrated that the anal gills were responsible for the active uptake of chloride from very dilute external media.

The anatomy of the larva of *A. aegypti* is shown in Text-fig. 1, which is taken from Wigglesworth (1933*b*). The mechanism of salt and water balance, as indicated by the work of Wigglesworth and of Koch, appears to be as follows. The larvae do not normally swallow the medium, except in so far as this is incidental to feeding. Water enters passively through the anal gills and salts are actively absorbed through these organs against the concentration gradient. Fluid—of unknown composition—is excreted by the Malpighian tubules and accumulates in the pyloric chamber; at intervals of a few minutes a drop of fluid passes down the intestine from the pyloric chamber to the rectum. In the rectum some of this fluid is resorbed and the rest is eliminated through the anus. The active uptake of chloride must involve osmotic work; but whether or not the activities of the Malpighian tubules and rectum involve osmotic work is unknown.

The larva of *A. detritus* shows essentially the same structure as that of *A. aegypti* except that the anal gills are reduced to small papillae. The osmotic relations are

very different. In fresh water the total osmotic pressure and chloride content are of the same order as in *A. aegypti*, but in various dilutions and concentrations of sea water, up to the equivalent of 6% NaCl, the total osmotic pressure and chloride rise steadily to values of 1·4 and 0·9% NaCl respectively (Beadle, 1939). The body surface is almost completely impermeable to water and salts, exchange taking place through the gut. The gut wall appears to be freely permeable to water and to glycerol but not to sucrose; but whether the salt water of the external medium is passively absorbed or whether there is differential absorption with the performance of osmotic work is not known. Beadle also showed that after ligatures had been applied between segments v and vi there was some increase in osmotic pressure and chloride content, which suggests that organs in the posterior part of the animal play some part in the regulatory process.



Text-fig. 1. A, anatomy of larva (semidiagrammatic): *ac*, anal canal; *ag*, anal gills; *c*, caeca; *int*, intestine (hindgut); *mg*, midgut; *mt*, Malpighian tubes; *pc*, pyloric chamber (hindgut); *pv*, proventriculus; *r*, rectum; *rs*, respiratory siphon; *tr*, one of the main tracheal trunks. The figures i, ii, etc., indicate the respective abdominal segments. B and C show cross-sections of the hindgut, B through the intestine (*int*), C through the rectum (*r*).—*From Wigglesworth* (1933*b*).

The ability of these larvae to regulate the composition of the haemolymph is therefore fairly clearly defined and a good deal is known of the intake side of the mechanism. But on the side of elimination we have no quantitative data at all. The work to be described in the present paper was undertaken with the object of making good this deficiency.

II. MATERIAL AND METHODS

Larvae of *A. aegypti* were bred from eggs and fed on a preparation of powdered dog-biscuit. In the third instar they were removed to clean media and thereafter starved. 'Distilled water larvae' and 'frog-ringer larvae' were reared in this way in the respective media. 'Double frog-ringer larvae' were reared with food in normal frog ringer and were later transferred to clean frog ringer of double strength. Cultures of 'frog-ringer larvae' survived well for 6–8 weeks; in the same period 50% of the 'distilled water larvae' died (the distilled water was continually renewed); 50% of the 'double frog-ringer larvae' died in 3–4 days.

Larvae of *A. detritus* were collected from a salt marsh near Walton-on-the-Naze,

Essex. Third and fourth instar larvae were kept in the laboratory in clean sea water and survived unfed for 6 weeks. All the work on *A. detritus* described in this paper was carried out on this single collection. Unfortunately, when a second collection was called for it was found that prolonged drought and an effective sea wall had temporarily eliminated the breeding pools. The work on this species was thus unavoidably curtailed.

Collections were made of the haemolymph and of the fluids from different regions of the gut. Very often only relatively small quantities, of the order of 0·01 cu.mm., were available. Such quantities were more than adequate for freezing-point measurements to be made by a method previously described (Ramsay, 1949), but it was not possible to determine chloride. The results here reported are restricted to measurements of total osmotic pressure, expressed in terms of the percentage concentration of an NaCl solution having the same freezing-point.

The regions of the gut studied were the rectum, the intestine, the midgut and the caeca; and a variety of methods of collection were adopted. In all cases it was necessary to restrain the movements of the larva, but at the same time it was desirable to avoid unnecessary interference with the workings of the viscera. For this reason anaesthetics were not used. The larva was secured by fine silk ligatures, one around the base of the respiratory siphon and the other around the neck; this second ligature, if desired, could be left sufficiently loose to permit swallowing. The larva was placed in a shallow layer of well-aerated medium and, although the tracheal system was occluded, activity was maintained up to 36 hr. or more. In well-aerated water sufficient gas exchange takes place through the general body surface to maintain life and activity. This was shown by Macfie (1917) for *A. aegypti*, and Beadle has shown that after ligation of the respiratory siphon in *A. detritus* the salt and water balance is undisturbed.

(*a*) *Rectal fluid* (R.F.). This was collected by inserting a cannula through the anal canal with the aid of a manipulator; in this way the fluid momentarily present in the rectum could be drawn up. By adding trypan blue to the external medium just before collection was made it could be shown that the external medium did not enter the cannula. Larger quantities could be collected over a longer period by tying a flared cannula* into the anal canal.

(*b*) *Intestinal fluid* (I.F.). It is possible to insert a moderately fine (100 μ diameter) cannula through the anus and rectum into the intestine. The circular muscle of the intestine is normally contracted and prevents rectal fluid from reaching the cannula. When a droplet of fluid is carried down by peristalsis from the pyloric chamber nearly all of it passes into the cannula.

* It is quite simple to prepare in the following way a small (100 μ diameter) cannula with a flared end which retains a ligature. Pyrex tubing is used, and is first drawn down to the required diameter and taper. The tapering point is broken off to the required length and is sealed off by being held in the edge of a coal-gas microflame (about 1·5 mm. height). The fine tube is then withdrawn from the flame and is connected to a compressed air supply at about one atmosphere pressure. The sealed end is again very carefully introduced into the edge of the flame. As it fuses a bubble forms and bursts. The remains of the bubble are broken off with fine forceps, leaving a jagged flared end. This end is then brought up to the edge of the flame (or to a nichrome wire heated bright red) and the jagged edges are allowed to round off. For these operations a binocular of medium power is desirable.

(*c*) *Midgut fluid* (M.F.). Owing to the loop in the intestine it is difficult to pass a cannula into the midgut from the anus. It is easier to reach the midgut by passing the cannula through the mouth, but the trick of doing this without injuring the larva was not discovered until a late stage in this work. In all the experiments of which the results are reported, collection from the midgut was made by a method involving dissection of the larva as described under (*d*) below.

(*d*) *Caecal fluid* (C.F.). It has not so far proved possible to insert a cannula into the caeca by way of any of the natural openings of the body, and it was therefore necessary to dissect out the gut and penetrate its wall. The larva was dried on cigarette paper and placed on a dry slide under a layer of liquid paraffin. A ligature was tied around the body between thorax and abdomen. The body wall was punctured and the haemolymph flowed out on the slide under the oil (at this stage the collection of the haemolymph was made). The tissues of the thorax were dissected away from the anterior region of the gut which was then impaled upon a fine silica 'freezing pipette' (Ramsay, 1949), connected with a screw plunger and filled with oil backed with mercury. When possible the point of the 'freezing pipette' was worked into the extremity of one of the caeca and a sample of fluid was drawn up. In other cases where this latter part of the operation did not succeed, the sample was taken from the lumen of the midgut in the region of the caeca. To collect from the extremity of a caecum is perhaps an unnecessary refinement in view of the periodic contractions of the caeca which cause exchange of fluid between the caeca and the neighbouring region of the midgut. The abdomen of the larva was then dissected, the midgut was impaled upon a second 'freezing pipette' and the sample was drawn up.

(*e*) *Haemolymph* (B.F.). This was collected directly into a 'freezing pipette' at the end of each experiment by drying the larva and puncturing it on a slide under oil, as described under (*d*) above.

It was possible to make all five collections from one and the same larva, but most experiments were conducted on a less ambitious basis. In every case a collection of haemolymph was made and this served as the standard of reference with which the other fluids were compared.

For purposes of histological examination material was fixed in Carnoy. Paraffin sections were cut at 10μ and stained in iron haematoxylin.

III. RESULTS

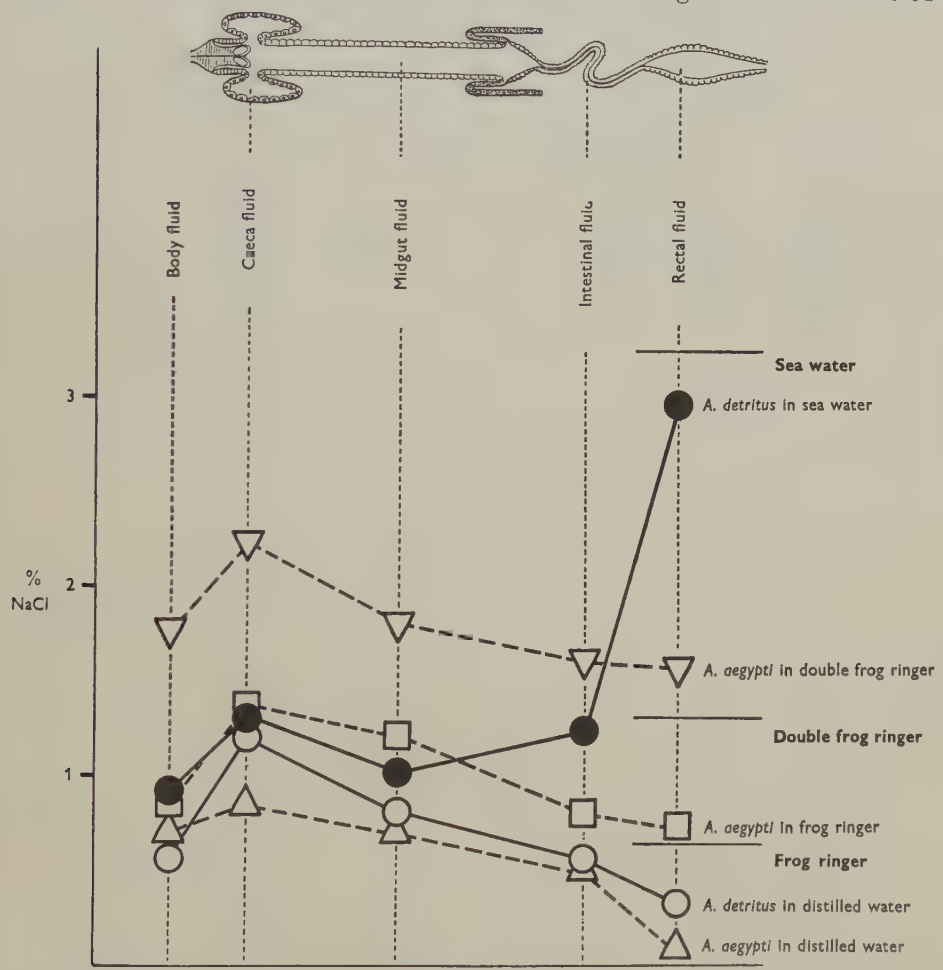
A. *Measurements of osmotic pressure*

The types of larvae studied may be placed in categories as follows: (1) *Aedes aegypti*, distilled water larvae; (2) *A. aegypti*, frog-ringer larvae; (3) *A. aegypti*, double frog-ringer larvae; (4) *A. detritus*, sea-water larvae; (5) *A. detritus*, distilled water larvae.

The larvae were kept for at least 48 hr. in the specified external medium before being used for experiment.

As described in the previous section, collections were made from the rectum, the

intestine, the midgut and the caeca. At least six collections (each from a different larva) were made from each position within each of the first four categories. A sample of haemolymph was collected from each larva. Results of the freezing-point determinations made on these collections are given in Tables 1–4. The differences in osmotic pressure between haemolymph and gut fluid have been subjected to the '*t*' test for significance, and the corresponding values of '*P*', taken from Fisher & Yates (1938), are given. $P<0{\cdot}05$ is considered to be significant and $P<0{\cdot}01$ is



Text-fig. 2. From the data of Tables 1–5. Each point represents the average value of osmotic pressure (in % NaCl) for one category of larva and one region of the gut; e.g. *A. aegypti*, frog-ringer larvae, rectal fluid, average of serials 3–8 = 0·735 % NaCl.

considered to be highly significant. Owing to the shortage of supply of *A. detritus* (for reasons already given) results of measurements in the fifth category, detailed in Table 5, amount to only two collections from each region and are insufficient for statistical treatment.

The data of Tables 1–5 are also shown graphically in Text-fig. 2.

Table 1. Aedes aegypti: *distilled water larvae*

Rectal fluid

Serial	R.F.	B.F.	Deviation +	Deviation −
27	0·06	0·56		0·50
28	0·13	0·57		0·44
29	0·08	0·54		0·46
30	0·07	0·63		0·56
33	0·07	0·62		0·55
34	0·02	0·61		0·59

Av. −0·52 % NaCl
$P < 0·001$

Intestinal fluid

Serial	I.F.	B.F.	Deviation +	Deviation −
1	0·59	0·56	0·03	
2	0·60	0·57	0·03	
14	0·54	0·52	0·02	
15	0·38	0·58		0·20
16	0·49	0·62		0·13
36	0·43	0·44		0·01

Av. −0·04 % NaCl
$P = 0·4 - 0·3$

Midgut fluid

Serial	M.F.	B.F.	Deviation +	Deviation −
27	0·68	0·56	0·12	
30	1·10	0·63	0·47	
33	0·79	0·62	0·17	
34	0·55	0·61		0·06
35	0·57	0·47	0·10	
47	0·56	0·67		0·11

Av. +0·155 % NaCl
$P = 0·3 - 0·2$

Caeca fluid

Serial	C.F.	B.F.	Deviation +	Deviation −
16	0·59	0·62		0·03
17	0·85	0·61	0·24	
27	0·79	0·56	0·23	
28	1·41	0·57	0·84	
29	1·02	0·54	0·48	
30	0·92	0·63	0·29	
33	0·70	0·62	0·08	
34	0·86	0·61	0·25	
35	0·56	0·47	0·09	

Av. +0·276 % NaCl
$P = 0·02 - 0·01$

Table 2. Aedes aegypti: *frog-ringer larvae*

Rectal fluid

Serial	R.F.	B.F.	Deviation +	Deviation −
3	0·77	0·82		0·05
4	0·69	0·79		0·10
5	0·64	0·80		0·16
6	0·89	0·93		0·04
7	0·71	0·82		0·11
8	0·71	0·76		0·05

Av. −0·085 % NaCl
$P < 0·001$

Intestinal fluid

Serial	I.F.	B.F.	Deviation +	Deviation −
9	0·79	0·79	0	
10	0·77	0·71	0·06	
11	0·92	0·88	0·04	
12	0·79	0·79	0	
13	0·79	0·84		0·05
37	0·79	0·97		0·18

Av. −0·022 % NaCl
$P = 0·6 - 0·5$

Midgut fluid

Serial	M.F.	B.F.	Deviation +	Deviation −
25	1·21	0·89	0·32	
37	1·08	0·97	0·11	
61	1·08	0·73	0·35	
62	1·77	0·94	0·83	
63	0·82	0·69	0·13	
64	1·36	0·60	0·76	

Av. +0·417 % NaCl
$P = 0·05 - 0·02$

Caeca fluid

Serial	C.F.	B.F.	Deviation +	Deviation −
25	1·04	0·89	0·15	
37	0·92	0·97		0·05
61	1·77	0·73	1·04	
62	1·58	0·94	0·64	
63	1·12	0·69	0·43	
64	1·87	0·60	1·27	

Av +0·58 % NaCl
$P = 0·05 - 0·02$

Frog ringer equivalent to 0·65 % NaCl.

Table 3. Aedes aegypti: *double frog-ringer larvae*

Rectal fluid

Serial	R.F.	B.F.	Deviation +	Deviation −
19	1·42	1·70		0·28
21	1·76	1·79		0·03
22	1·58	1·85		0·27
26	1·78	1·80		0·02
32	1·49	1·88		0·39
56	1·57	1·76		0·19

Av. −0·197 % NaCl
$P = 0{\cdot}05 - 0{\cdot}02$

Intestinal fluid

Serial	I.F.	B.F.	Deviation +	Deviation −
18	1·66	1·64	0·02	
20	1·53	1·73		0·20
57	1·43	1·98		0·55
58	1·78	1·58	0·20	
59	1·43	1·54		0·11
60	1·89	1·63	0·26	

Av. −0·063 % NaCl
$P = 0{\cdot}7 - 0{\cdot}6$

Midgut fluid

Serial	M.F.	B.F.	Deviation +	Deviation −
19	1·70	1·70	0	
23	1·68	1·77		0·09
24	1·88	1·60	0·28	
26	2·33	1·80	0·53	
31	1·51	1·51	0	
32	1·88	1·88	0	

Av. +0·12 % NaCl
$P = 0{\cdot}3 - 0{\cdot}2$

Caeca fluid

Serial	C.F.	B.F.	Deviation +	Deviation −
20	2·44	1·73	0·71	
21	2·68	1·79	0·89	
22	2·32	1·85	0·47	
23	2·71	1·77	0·94	
24	1·86	1·60	0·26	
31	1·76	1·51	0·25	
32	1·98	1·88	0·10	

Av. +0·517 % NaCl
$P = 0{\cdot}01 - 0{\cdot}001$

Double frog ringer equivalent to 1·32 % NaCl.

Table 4. Aedes detritus: *sea-water larvae*

Rectal fluid

Serial	R.F.	B.F.	Deviation +	Deviation −
38	2·39	1·07	1·32	
39	2·14	0·83	1·31	
40	2·32	0·82	1·50	
41	3·85	0·87	2·98	
42	3·55	0·93	2·62	
43	3·84	1·00	2·84	
44	2·97	0·91	2·06	

Av. +2·09 % NaCl
$P < 0{\cdot}001$

Intestinal fluid

Serial	I.F.	B.F.	Deviation +	Deviation −
48	1·03	0·84	0·19	
49	1·28	0·97	0·29	
50	1·18	0·98	0·20	
51	1·57	0·90	0·67	
52	1·04	0·96	0·08	
53	1·38	0·97	0·41	

Av. +0·31 % NaCl
$P = 0{\cdot}02 - 0{\cdot}01$

Midgut fluid

Serial	M.F.	B.F.	Deviation +	Deviation −
38	1·20	1·07	0·13	
39	0·83	0·83	0	
41	0·89	0·87	0·02	
42	0·96	0·93	0·03	
43	1·25	1·00	0·25	
44	1·14	0·91	0·23	
45	0·98	0·90	0·08	
46	1·00	0·96	0·04	

Av. +0·10 % NaCl
$P = 0{\cdot}05 - 0{\cdot}02$

Caeca fluid

Serial	C.F.	B.F.	Deviation +	Deviation −
41	1·72	0·87	0·85	
42	1·68	0·93	0·65	
43	1·27	1·00	0·27	
44	1·17	0·91	0·26	
45	0·96	0·90	0·06	
46	1·14	0·96	0·18	

Av. +0·38 % NaCl
$P = 0{\cdot}05 - 0{\cdot}02$

Sea water equivalent to 3·28 % NaCl.

Table 5. Aedes detritus: *distilled water larvae*

Serial	B.F.	R.F.	I.F.	M.F.	C.F.
54	0·55	0·38	0·55	1·02	1·21
55	0·59	0·28	0·59	0·62	—

The most important points which emerge are the following: (*a*) the caeca fluid is always hypertonic (to the haemolymph); (*b*) the midgut fluid is either isotonic or hypertonic; (*c*) the intestinal fluid is isotonic, except in the case of *A. detritus* in sea water, where it is hypertonic; (*d*) the rectal fluid is hypotonic in both *A. aegypti* and *A. detritus* when the larvae are in distilled water. In *A. aegypti*, when the larvae are in frog ringer or double frog ringer, the rectal fluid is still slightly hypotonic. In *A. detritus*, when the larvae are in sea water, it is strongly hypertonic.

From these results certain main conclusions may be drawn at once.

(1) In *A. aegypti*, in fresh water, osmotic work is carried out in the rectum with the effective dilution of the fluid which enters from the intestine.

(2) In *A. detritus*, in sea water, osmotic work is carried out in the rectum with the effective concentration of the fluid which enters from the intestine.

(3) In *A. aegypti* there is no evidence that the rectum can effect concentration of the fluid which enters from the intestine.

(4) In *A. detritus* there is evidence, from two examples only, that the rectum can effect dilution of the fluid which enters from the intestine.

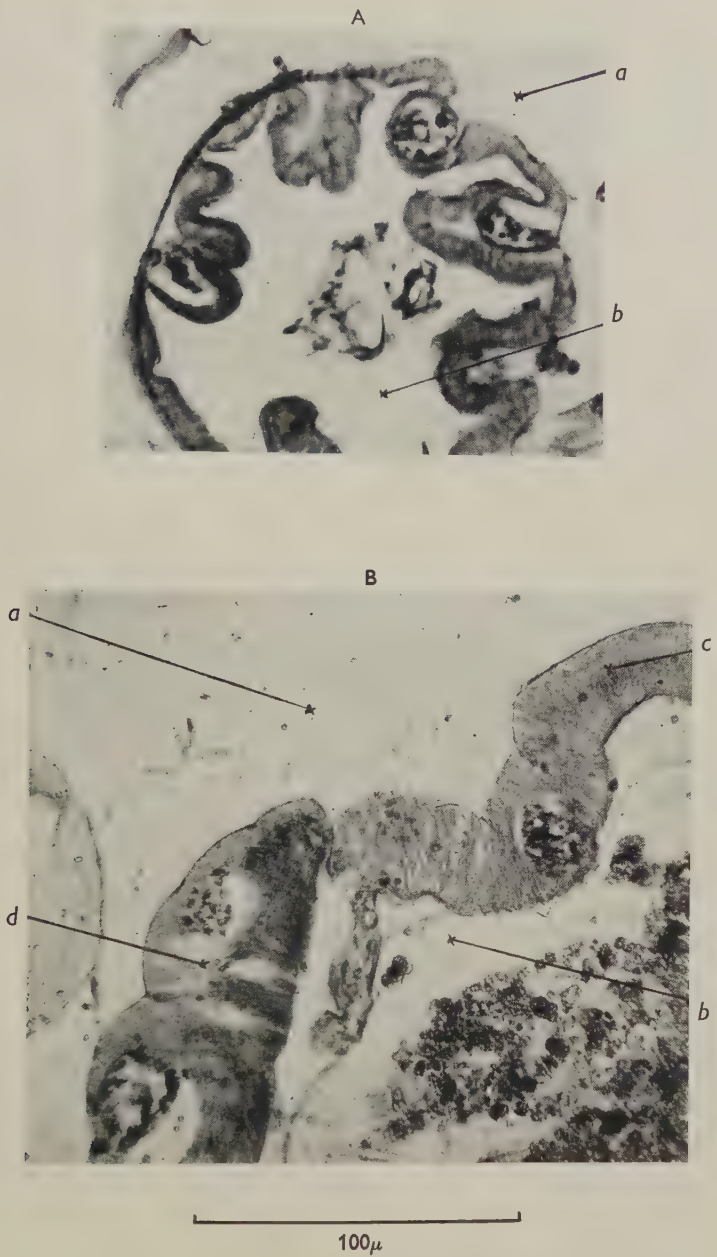
These main conclusions, and others of a more tentative nature, are further discussed in section IV.

B. *Histology of the rectal epithelium*

In view of the conclusions reached in the preceding subsection about the functions of the rectum, it is obviously of interest to know whether there are any differences in the structure of the rectum in *A. detritus* as compared with *A. aegypti*, to which the observed differences in function may be related.

The histology of the rectal epithelium of *Aedes* larvae does not appear to have been studied in detail. Wigglesworth (1933*b*)—see also Text-fig. 1—gives a small-scale cross-section and refers to the 'relatively large epithelial cells'. The investigation reported here is admittedly incomplete, but seems to indicate a possible correlation between histological structure and physiological function.

The rectal epithelium of *A. aegypti* is fairly deep and folded, with large nuclei but without distinguishable cell boundaries. The cytoplasm shows evidence of striation and appears to be more dense along the margin adjoining the lumen of the rectum. This dense border appears to be continuous and interposes itself between the nuclei and the lumen. Very often the nuclei appear as it were to be forced out of the striated cytoplasm altogether and to lie within its folds; that is to say, the nuclei lie on the margin of the epithelium which adjoins the body cavity—see Pl. 1 A. Six specimens of *A. aegypti* have been examined in serial sections, and in all



A, transverse section through rectum of *Aedes aegypti*. *a*, body cavity; *b*, lumen of rectum. B, transverse section (somewhat oblique) through rectum of *A. detritus*, showing transition from anterior epithelium, *c*, to posterior epithelium, *d*. Both photographs are to the same scale.

of them the epithelium appears to be uniformly of this type throughout the rectum.

A. detritus is a larger species than *A. aegypti*, and in addition the rectum of *A. detritus* appears relatively somewhat larger and better supplied with tracheae. As displayed in dissection there is nothing to suggest any division of the rectum into two regions. But when sections are examined it is clear that two kinds of epithelium are present. The posterior portion, extending from the anal canal to about the middle of the rectum, is lined with an epithelium which, although deeper than the epithelium of *A. aegypti*, appears to have the same character, i.e. a layer of dense cytoplasm on the side next to the lumen and the nuclei on the side next to the body cavity. The anterior portion, extending from the middle of the rectum to the intestine, is lined with an epithelium which is likewise deep and folded, and shows evidence of striation; but on the whole its cytoplasm is less dense, and if anything the density is greater on the side away from the lumen. The nuclei may be found in any position: on the side of the lumen, away from the lumen or in the middle. Further, the transition between these two types of epithelium is quite sharp; the anterior portion is overlapped and enclosed by the posterior portion for a short distance over which the anterior portion thins rapidly and projects inwards in the form of a small flap—see Pl. 1 B. Four specimens of *A. detritus* have been sectioned and all show the two types of epithelium as described above.

The marked difference in size and thickness of the epithelium in the two species makes it difficult to claim with certainty that the epithelium of *A. aegypti* belongs to one or other of the two types of epithelium in *A. detritus*. It is clear, however, that the epithelium of *A. aegypti* bears much more resemblance to the posterior than to the anterior epithelium of *A. detritus*, and the interpretation placed upon these results is that *A. detritus* possesses in the anterior portion of the rectum a special type of epithelium which is not represented in *A. aegypti*.

IV. DISCUSSION

The conception of the mechanism of salt and water balance in *A. aegypti*, as indicated by the work of Wigglesworth and of Koch, has been outlined in section I. We are now in a position to add that the fluid passing down the intestine is isotonic with the haemolymph, and that the rectal epithelium carries out osmotic work in rendering this fluid hypotonic before it is eliminated. Whether this is brought about by resorption of salts or by secretion of water is not yet demonstrated; but Wigglesworth's (1933*b*) observations suggesting a resorption of fluid in the rectum do not support the second alternative. Attempts made during the course of the present work to demonstrate changes of volume in the rectum were inconclusive.

Mainly on the basis of experiments in which larvae were fed on solid trypan blue, Wigglesworth suggested that fluid is secreted into the lumen of the gut by the cells of the posterior midgut region, and that this fluid passes forward to the caeca where it is resorbed. These experiments have been repeated, using trypan blue and also phenol red which is even more instructive.

After a larva has been allowed to swallow a saturated solution of phenol red the gut has a characteristic appearance. The caeca appear red (pH 7·6–8·0), the midgut is crimson (pH > 8·4), the region of the pyloric chamber is orange-red (pH 7·4–7·8) and the rectum is yellow (pH < 7·2).* When a droplet passes into the rectum from the intestine the colour change is complete in about a minute.

About a day later most of the dye has disappeared from the gut; what remains is to be seen in the caeca and extending some little distance down the midgut, a distribution which seems to indicate a general forward movement of the fluid in the midgut.

The orange-red fluid of the pyloric chamber is presumably midgut fluid mixed with fluid excreted by the Malpighian tubules. The excretion by the Malpighian tubules could of itself make available a supply of fluid to support the forward movement in the midgut, but the change of colour in the middle region of the midgut indicates some activity on the part of the cells of this region. The osmotic pressure measurements show that the midgut contents are somewhat hypertonic to the haemolymph. It is possible that osmotically active substances are secreted into the lumen and that water follows passively. This implies that the gut wall is readily permeable to water, and a simple experiment demonstrates that this is, in fact, the case. A larva is placed in a 2M-sucrose solution saturated with phenol red. The larva swallows this medium, and after a few minutes the dye is seen in the anterior part of the midgut. The neck and anal canal are then ligatured to prevent any further exchanges with the external medium. In 20 min. the gut is seen to have become enormously distended, almost filling the body, and it is obvious that the fluid in the gut has increased at the expense of the haemolymph.

The evidence that the fluid of the midgut is resorbed in the caeca, combined with the evidence of higher osmotic pressure in the caeca, must be taken to indicate that the cells of the caeca are capable of osmotic work. But it does not appear that this internal circulation of water from haemolymph → midgut → caeca → haemolymph plays any direct part in the osmotic relations between the larva and the external medium.

Since in *A. aegypti* the fluid passing down the intestine is isotonic with the haemolymph, it does not appear likely that in this species the Malpighian tubules have more than a passive role in osmotic (as distinct from ionic) regulation. In *A. detritus*, on the other hand, the fluid collected from the intestine is definitely hypertonic to the haemolymph and to the midgut fluid. Although this observation suggests osmotic activity on the part of the Malpighian tubules it is to be accepted with caution for the following reason. It was observed in *A. detritus* that when a small droplet passing down the intestine reached the rectum there was sometimes an incipient retro-peristalsis, the posterior portion of the intestine becoming momentarily distended with fluid from the rectum. (Such retro-peristalsis was never observed in *A. aegypti* unless the anus was obstructed and an excess of fluid had accumulated in

* In view of the errors inherent in the use of indicators for pH work the figures should not be taken to represent the true pH of the gut contents. The figures are intended to describe the colour of the indicator as it would appear in standard buffer solutions.

the rectum.) Although the cannula was thrust as far up the intestine as possible, it may have happened that some rectal fluid found its way into the cannula in this way. Beadle was inclined to attribute an active excretion of salts to the Malpighian tubules on the basis of his experiments with ligatures, but since the ligatures he applied must also have eliminated the activity of the rectum, the part played by the Malpighian tubules remains uncertain.

When this investigation was nearing completion my attention was drawn to the work of Boné & Koch (1942) on the larva of *Limnophilus flavicornis*. Boné & Koch collected fluids from the anus and from the intestine and determined their chloride content. They assumed that the intestinal fluid was derived solely from the excretion of the Malpighian tubules; this may well be true, but it is not to be forgotten that the intestine is in free communication with the midgut and may receive fluid from the midgut as well as from the Malpighian tubules. It was shown that when the larva was placed in an external medium of 0·1 % NaCl the chloride content of the intestinal fluid was greater than that of the haemolymph; when the larva was placed in 0·001 % NaCl the chloride content of the intestinal fluid was less than that of the haemolymph, and further reduction of the chloride content was shown to occur in the rectum. In so far as they indicate a reduction in the concentration of osmotically active substances in the rectum the results obtained with *Aedes aegypti* are in agreement with the results of Boné & Koch on *Limnophilus*. At first sight it would appear that there is disagreement as to the part played by the Malpighian tubules. But there is no reason to suppose that the differences in chloride content as between the intestinal fluid and haemolymph in *Limnophilus* are incompatible with equality in total osmotic pressure; it may be that the chloride/non-chloride ratio of osmotically active substances is capable of being varied in the Malpighian tubules, while the total osmotic pressure remains the same as that of the haemolymph. On the other hand, in view of the anatomical and histological differences known to exist in the Malpighian tubules of different orders of insects a radical difference in the physiological capability of the Malpighian tubules in *Limnophilus* as compared with *Aedes* need not be surprising. This uncertainty serves to emphasize the desirability of determining both chloride content and total osmotic pressure in the case of animals whose body fluids contain a high proportion of 'organic' solutes, and it is unfortunate that there is no method suitable for chloride determination on the small quantities of fluid obtainable from the gut of the mosquito larva.

As far as differences in total osmotic pressure (as distinct from possible differences in chemical composition) are concerned the rectum is the organ in the mosquito larva which plays the most important part in regulation. It remains for us to consider whether the activity of the rectum—and of other organs such as the anal gills—is sufficient to account for the observed ability of the larvae to survive in various media. In *A. detritus* the reduced anal gills are believed to be without function; the larva swallows the external medium and therefore it must be able, broadly speaking, to eliminate a fluid of the same osmotic pressure if it is to maintain the constancy of the haemolymph. Reference to Table 4 shows that in three cases out of seven the rectal fluid was substantially more concentrated than the external medium,

showing that in normal sea water the rectum operates with some margin of safety. How it fares in more concentrated sea water, equivalent to 6% NaCl, has not been studied.

The case of *A. aegypti* is rather more difficult, because of the parenteral absorption of water and salts through the anal gills. But when the animal is kept in a current of distilled water the amount of salt absorbed by the anal gills must surely be insignificant. Under these conditions the fluid collected from the rectum has an osmotic pressure equivalent to 0·07% NaCl (Table 1). No doubt some part of this is attributable to nitrogenous excretory matter, but the possibility of some salts being present can hardly be denied. Wigglesworth (1933*b*) estimates that the volume of fluid eliminated by a larva living in tap water 'certainly does not exceed the cubic capacity of two anal gills per hour'; this means that the larva will eliminate something like 20% of its own volume in 24 hr. Wigglesworth has also shown (1938) that when the larva is placed in distilled water the chloride content of the haemolymphs falls steadily to 0·05% NaCl in 8 days and thereafter remains constant. Even if the chloride content of the rectal fluid is of the order of 0·005% NaCl, as found for *Limnophilus* by Boné & Koch, the whole chloride content of the larva might be lost in 4–5 weeks. There is, however, some evidence that during prolonged starvation in a current of distilled water there is reduction in the volume of fluid eliminated by *Aedes aegypti*. Animals taken for examination often show a more or less continuous column of faeces extending through the intestine to the rectum. When observed on a slide, especially if the larva's movements are restrained by the pressure of a cover-slip or by ligatures, the elimination of faeces begins almost at once, and after the solid contents of the hindgut have been evacuated the characteristic regular passage of drops down the intestine can be seen. It is difficult to believe that the solid column of faeces could have been established at a time when droplets of fluid were regularly passing down the intestine. This observation suggests that when the larva is undisturbed the elimination of faeces and fluid from the anus is in abeyance, that if fluid continues to be excreted by the Malpighian tubules it passes forwards to the caeca and that the intake of water through the anal gills is reduced.

Wigglesworth (1933*b*) considered the possibility that the passive entry of water through the anal gills might be actively resisted by a mechanism analogous to that which is known to exist in frogs' skin. But his experiments showed that when elimination of fluid was prevented by a ligature between segments vi and vii the posterior part of the animal swelled up and might even burst, and he therefore recognized the lack of any evidence for active control of water intake. Nevertheless, the idea that there may be an active resistance to the entry of water at the anal gills and that this resistance readily breaks down when the larva is harassed is one which should not be discarded prematurely.

The histological observations reported in section III B are not in sufficient detail to merit extensive discussion. It is interesting, however, to have some clue as to the possibility of identifying certain physiological processes of osmotic regulation with discernible histological characters. Pagast (1936) has observed differences in the appearance of the Malpighian tubules of *A. aegypti* according to the medium in

which the larvae were kept. Harnisch (1934) has also applied histological methods to the problem of osmotic regulation in *Chironomus thummi*, but reaches conclusions which are difficult to accept. Progress with the same general problem is reported by Pettengill & Copeland (1948) and by Copeland (1948), who have identified the chloride-secreting cells in the gills of the fish *Fundulus*. Mosquito larvae would appear to provide promising material for an extension of these studies.

V. SUMMARY

1. The processes of osmotic regulation in the larvae of *Aedes aegypti* and of *A. detritus* have been studied by determination of the freezing-point of samples of fluid collected from different parts of the gut.

2. In *A. aegypti*, kept in fresh water (its normal environment), the fluid passing down the intestine to the rectum is isotonic with the haemolymph. In the rectum it becomes strongly hypotonic before being eliminated.

3. In *A. detritus*, kept in sea water (its normal environment), the opposite process is observed, the fluid in the rectum becoming hypertonic to the haemolymph and approximately isotonic with the external medium before being eliminated.

4. In *A. detritus*, which is able to live in dilute media as well as in sea water, the only two specimens from fresh water available for examination were found to have the rectal fluid hypotonic to the haemolymph.

5. The ability of *A. detritus*, not possessed by *A. aegypti*, to produce an hypertonic fluid in the rectum is tentatively associated with a region in the anterior part of the rectum and lined with an epithelium distinctly different from that in the remainder of the rectum. This anterior region has not been found in *A. aegypti*.

I am greatly indebted to Dr V. B. Wigglesworth for reading the manuscript of this paper and for most valuable discussion. I wish to thank Sir Rickard Christophers for providing me with the eggs of *A. aegypti* and for advising me on the method of rearing this species; and I also wish to thank him and Mr P. G. Shute for going to great trouble to help me obtain supplies of *A. detritus*. Nearly all the freezing-point measurements and preparation of sections were carried out by my assistant, Miss J. Gukenbiehl.

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THE PHYSIOLOGY OF CARBON DIOXIDE TRANSPORT IN INSECT BLOOD

PART I. THE FORM OF CARBON DIOXIDE PRESENT IN *GASTROPHILUS* LARVA BLOOD

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(With Two Text-figures)

INTRODUCTION

In contrast to the numerous studies on the analytical biochemistry of insect blood—see Maluf (1939) and Timon-David (1945) for a summary of the literature—the function of the blood in the physiology of insects has remained comparatively neglected. In this paper an investigation has been made of the respiratory function of the blood in the larva of the fly, *Gastrophilus intestinalis* de Geer (the horse bot fly), with special reference to carbon dioxide transport.

The larva of *Gastrophilus* is of particular interest for the present type of investigation. The adult and pupal stages are free living, whereas the larva is parasitic in the alimentary tract of the horse. The fully grown 3rd instar larva is attached to the mucous membrane of the stomach, where for a period of 7–8 months it remains in a quiescent state which may be likened to diapause. Its respiratory system is well developed and highly specialized, in order that an aerobic mode of life may continue in an environment very poor in oxygen but rich in carbon dioxide. These larval respiratory adaptations have been discussed by Keilin (1944) and Keilin & Wang (1946), so that in this work attention need only be drawn to the possession of haemoglobin, of a type which is peculiar to *Gastrophilus* and localized principally in the tracheal organ—a mass of very large cells attached to four short tracheal trunks at the posterior end of the body—and to the presence of an efficient spiracular closing mechanism. It will be shown below that the larva is not only adapted to an environment deficient in oxygen, but that in its relation to carbon dioxide it shows some equally specialized physiological features.

The conditions under which CO_2 transport occurs in insect blood are somewhat simpler than those existing in the circulatory system of the higher types of animals. Thus the blood of insects, with but very few exceptions (e.g. *Chironomus* larvae) contains no respiratory carrier such as haemoglobin or haemocyanin. The amount of oxygen carried by the blood is no more than can be accounted for by solubility considerations (Bishop, 1923; Babers, 1941), and hence complications due to oxygenation or reduction of the blood (the so-called Haldane effect) need not be considered so far as CO_2 transport is concerned. Again, in Vertebrates the blood is a heterogeneous system consisting of cells and plasma, whereas in insect blood the

number of cells or haemocytes is generally small, numbering only some tens of thousands per cu.mm. (Tauber & Yeager, 1935, 1936), so that compared with an average red cell count they may be ignored, and the blood be treated as a homogeneous fluid.

The published data show that solubility considerations are not sufficient to account for the amounts of CO_2 present in the blood of different insects. Thus, Bishop (1923) found 29·2 vol. % CO_2 in bee-larva blood, and Babers (1941) reported 10·03 vol. % CO_2 for the blood of the larva of the southern armyworm (*Prodenia eridania*). In both these cases it may be calculated that only about one-fifth of the total CO_2 was in solution. It is also highly probable that of the 9·24–81·1 vol. % CO_2 present in the blood of various other insect species (Florkin, 1937; Drilhon & Busnel, 1937), only a similar small fraction of the total CO_2 could be dissolved. It is clear, therefore, that gaseous CO_2 produced by insects' tissues is bound in some form in the blood, prior to its reconversion into the gaseous state and subsequent removal from the body by diffusion.

Some of the salient features concerning present views on CO_2 transport by vertebrate blood will now be briefly summarized, in order to facilitate a comparison with the data presented in this paper for insect blood.

There are six forms in which carbon dioxide may be transported in the blood, viz. dissolved anhydrous CO_2, carbonic acid-H_2CO_3, bicarbonate-$BHCO_3$, carbamate-$R.NH.COOH$, and Y-bound CO_2. These forms are interrelated in the following manner (Roughton, 1935).

When gaseous carbon dioxide dissolves in pure water, part of it goes into physical solution to an extent determined by the partial pressure of the gas, and the remainder reacts with water to form carbonic acid, as represented by the equation

$$H_2O + CO_2 \rightleftharpoons H_2CO_3 \quad \text{or} \quad \frac{[CO_2][H_2O]}{[H_2CO_3]} = K, \qquad (1)$$

where square brackets denote concentration. The equilibrium constant K is such, that the reaction proceeds to the right only to the extent of 1% or less, the bulk of the gas being in the form of anhydrous CO_2. Since the concentration of water $[H_2O]$ may be considered a large and constant quantity, the proportion of the anhydrous CO_2 in the hydrated form may be considered constant, with the result that the term $[H_2CO_3]$ may be used to represent all the uncombined free CO_2, instead of the more cumbersome term $[CO_2]+[H_2CO_3]$.* However, in most biological fluids there is always an excess of base present (base, as used in the present sense, denotes kations, of which Na^+, K^+, Ca^{++} and Mg^{++} are the most important), and under these conditions the base immediately reacts with carbonic acid to form the highly ionized bicarbonate salts. All the base of the blood which is not combined with acids stronger than carbonic acid is available for bicarbonate formation, and it is the quantity of available base which largely limits the amount of bicarbonate that can be formed in the blood. The presence of available free base, therefore, greatly increases the amount of CO_2 that can be carried by the blood.

At a pH above 8, significant amounts of carbonate commence to be formed; but carbonate

* This convention was originally adopted by Van Slyke before the value of the equilibrium constant K and the formation of carbamate was known. Although no errors are involved if it is correctly employed, the use of this convention is now irrational, and it is presented here for the sake of simplicity.

formation is negligible in the physiological pH range for vertebrates, and is practically non-existent in insect blood, the pH of which is generally below 7.

The relation between free CO_2 and bicarbonate, i.e. $[H_2CO_3]$ and $[BHCO_3]$, is given by the well-known Henderson-Hasselbalch formula

$$pH = pK_1' + \log \frac{[BHCO_3],}{[H_2CO_3]} \qquad (2)$$

where pK_1' is the negative logarithm of the apparent first dissociation constant for carbonic acid. As pointed out by Hastings, Sendroy & Van Slyke (1928), this term actually includes the true dissociation constant of the more acid hydrogen atom of carbonic acid, the activity coefficient of the HCO_3^- anion, the equilibrium constant for the reaction $H_2O + CO_2 \rightleftharpoons H_2CO_3$ and the dissociation constant of $BHCO_3$ should the latter be incompletely dissociated. Despite its composite nature, the pK_1' has a quite definite value; if this value is known, then given any two terms in equation (2), the third term may be calculated. Conversely, the pK_1' may be calculated if the three other terms are known, assuming that the whole of the CO_2 of the blood is either free CO_2 or bicarbonate.

The uncatalysed rate of the reaction $H_2O + CO_2 \rightleftharpoons H_2CO_3$ is very slow, and takes many minutes to reach equilibrium. However, this rate is enormously increased in presence of the specific enzyme carbonic anhydrase which is present in the blood of vertebrates and many invertebrates. A study of the kinetics of the above reaction led Henriques (1928) to first suggest that part of the bound CO_2 of mammalian blood was not in the form of bicarbonate, but was in some way bound to haemoglobin. During the course of their work on carbonic anhydrase, Meldrum & Roughton (1934) confirmed and extended Henriques's observations, and in turn suggested that this non-bicarbonate CO_2 was in the form of a carbamino compound. Such carbamino compounds are formed when CO_2 reacts with a free $-NH_2$ group of a protein, amino-acid or amine according to the reaction

$$CO_2 + R.NH_2 \rightleftharpoons \underset{\text{(carbamino compound)}}{R.NH.COOH} \rightleftharpoons R.NHCOO^- + H^+. \qquad (3)$$

Carbamino (or carbamate) formation is a rapid spontaneous reaction, promoted by a decrease in temperature and a pH of increasing alkalinity. It does not occur at pH 6 or below. Carbamino formation in blood has been reviewed by Roughton (1935, 1943), but its quantitative physiological importance is still a matter of controversy (e.g. Wyman, 1948).

Ferguson & Roughton (1934) raised the question as to whether the whole of the bound CO_2 in blood could be accounted for as bicarbonate and carbamate. A survey of the literature showed that, in addition to the forms discussed above, there was strong evidence for the existence of CO_2 bound in some unknown manner, and this fraction they termed Y-bound CO_2.

MATERIAL

The blood used for the present study was obtained from *Gastrophilus* larvae which were supplied in large numbers by the local abattoir; still attached to the stomach, they were transported to the laboratory in a thermos flask containing either warm water at 38° C. or water and ice at 0° C. For details as to the morphology and biology of *Gastrophilus* larvae, reference may be made to the papers of Dinulescu (1932) and Keilin (1944).

About 30 min. after their removal from the stomach of an infected horse, the larvae arrived in the laboratory, where they were washed with either warm or ice-cold water, detached from the mucosa with forceps, rinsed in two or three changes of water and dried between sheets of filter-paper.

Blood was obtained from the insects by making a small incision at the anterior end and allowing the haemolymph to drain through a gauze-covered funnel into a tube standing in either warm water or ice. When blood that had not been exposed to air was required, the larvae were bled by making a similar incision while they were immersed under a layer of 3 cm. of liquid paraffin in a narrow tube.

Examination of a thick layer of *Gastrophilus* blood with the microspectroscope shows faint bands of oxyhaemoglobin. Its concentration is so low, however, that for all practical purposes the haemoglobin in the blood may be ignored; but in crushed or moribund larvae, or under certain other abnormal conditions, the haemoglobin leaks out from the tracheal organ causing both the blood and the whole larva to become distinctly red. For this reason, any specimen whose blood showed the slightest reddish tinge was rejected.

METHODS

For saturation of the blood with various tensions of CO_2, the special small-sized tonometers of about 60 ml. volume described by Barcroft (1934), and the procedure for their use as given by this author, were employed. About 1 ml. of blood from a pooled blood sample was added to each tonometer, four of which were used at a time. As is frequently the case with insect haemolymph, *Gastrophilus* blood exposed to the air soon turns dark due to the formation of melanin. This reaction does not occur in the absence of oxygen, and accordingly the tonometers containing the blood samples were repeatedly evacuated and filled with oxygen-free nitrogen. By this procedure melanin formation was largely, but not entirely, prevented. As *Gastrophilus* blood does not clot, it was unnecessary to add any anti-coagulant.

The tonometers having been filled with CO_2 to approximately the partial pressures required, the blood they contained was equilibrated with the gas mixture in the usual way. Preliminary experiments showed that much longer times were necessary for equilibration than is the case for mammalian blood. At 38° C. about 90 min. was found to be adequate, while at room temperature (*c.* 16° C.) at least 2 hr. was required for equilibration. Subsequently, with the tonometer in a vertical position in the water-bath and the tip of the innermost Van Slyke pipette just above the water, the blood was allowed to rise up the pipette and directly into the capillary of a special micro-glass electrode, the tip of which was in contact with that of the Van Slyke pipette. The blood sample in the electrode served for measurement of the pH and the 0·4 ml. sample in the pipette for analysis of total CO_2 with the Van Slyke manometric apparatus. The gas phase in the tonometer was analysed for its CO_2 content by means of the Haldane gas analysis apparatus.

The micro-glass electrode used in conjunction with the Cambridge Instrument Co. pH meter was similar to the one described by Michaelis (1936). It had a small rubber teat at its upper end, and a capacity of *c.* 0·2 ml. Contact with the saturated calomel half-cell was made through a drop of saturated KCl on a paraffined slide and a capillary agar bridge. The electrode was standardized against potassium hydrogen phthalate pH 3·97 before each reading.

Measurement of the CO_2 content of the tissues

No published method for the measurement of the CO_2 content of the tissues (e.g. Anrep, Ayadi & Talaat, 1936; Danielson & Hastings, 1939) was suitable for use with *Gastrophilus*, but the following simple procedure gave satisfactory results.

The larvae were bled, quickly cut open, and the last traces of blood removed with a jet of ice-cold *c.* 0·1% NaOH. They were then quickly dried with filter-paper and placed in a test-tube containing a known volume of 0·1% NaOH. By weighing the tube before and after the addition of the larvae, the weight of the larvae was obtained. The test-tube, ground on the inside at the bottom end, served as a mortar for the homogenizing apparatus of Potter & Elvehjem (1936). The larvae were ground ('homogenized') for about a minute with an electrically driven glass pestle, after which time a finely divided tissue pulp was obtained which could be pipetted easily into the extraction chamber of the Van Slyke apparatus. By the above treatment all the larval tissues were scraped off from the cuticles and ground, but the cuticle itself was unaffected. After the analysis, therefore, the entire cuticles were taken out, rinsed in distilled water, dried on filter-paper and weighed. This weight was substracted from the weight of the whole larvae in order to calculate the weight of tissue in the 0·5 ml. sample analysed. It was assumed that the density of the larvae was the same as that of the soda.

The 0·1% NaOH, if freshly made up in boiled distilled water, could be used directly for a determination; however, as a result of allowing the solution to stand, smaller blanks were obtained—a blank determination on the soda was always made concurrently with the unknown—by a few minutes' preliminary evacuation on the water pump before use to remove CO_2.

Carbamino formation

For estimation of carbamino formation by *Gastrophilus* blood, the method employed by Meldrum & Roughton (1934) of measuring the rate of CO_2 uptake by blood under various conditions was followed. The apparatus used was a modification of that described by Stadie & O'Brien (1935), substituting for their pipette the rapidly shaken glass boat of Meldrum & Roughton. A large Kjeldahl flask served as a CO_2 reservoir, and a smaller one as a compensating vessel for the boat; a three-way tap connected one side of the manometer to either the reservoir or the suction pump. In making a run, the procedure of Roughton & Booth (1938) was followed with but slight modification, and was briefly as follows:

2 ml. of phosphate buffer, the pH and molarity of which were varied in different experiments (the rate of CO_2 uptake is influenced by alteration of either of these), 2 ml. of blood and a drop of caprylic alcohol to prevent excessive foaming were placed in the glass boat. The CO_2 reservoir was alternately evacuated and filled a number of times with pure cylinder CO_2, to a final pressure of exactly one-fifteenth of an atmosphere as measured on the Hg manometer. Then, by means of the three-way tap, the boat and its contents were evacuated for 5 min. at the water pump, and for a further 3 min. while the boat was shaken. The shaking was now stopped, the

pressure adjusted to one-tenth of an atmosphere and the boat connected to the CO_2 reservoir. The resulting pressure drop on the Hg manometer was noted, and exactly 1 min. allowed for any disturbances resulting from the entry of the CO_2 into the boat to settle down. (The amount of CO_2 absorbed during this minute is negligible.) Shaking was then recommenced, and readings taken every 5 sec. for the first minute, and at 30 sec. intervals thereafter. In any one series of runs the final pressure drop on the Hg manometer, and hence the CO_2 tension in the boat, was reproducible to within a $\frac{1}{5}$ mm. of Hg.

The same apparatus was employed for measuring the rate of CO_2 output from the blood, a single sample frequently serving for both types of experiment. The only alteration in the apparatus necessary for the latter runs was to open the manometer on the opposite side to the boat, and to close the connecting tube between the two limbs of the manometer. The temperature of the bath for all manometric experiments was 0° C.

RESULTS

(*a*) *Hydrogen-ion concentration.* The pH of *Gastrophilus* blood as drawn from the larva without exposure to air at room temperature varies from 6·74 to 6·90 with a mean of 6·80. This value is fairly general for the pH of insect blood (see Waterhouse, 1940, for a summary of the literature on the pH of insect blood). On allowing the blood to stand, the pH first increases slightly, but on further standing for a period of 2–3 hr. it commences to decrease. These changes are quite small, amounting to not more than 0·1–0·2 pH unit. They may be explained by the facts that there is at first a slow loss of CO_2 tending to make the blood more alkaline, followed by lactic-acid formation as a result of which the pH decreases. It is possible that this increase in lactate is due to bacterial infection. Blowing off the bulk of the CO_2 by passing a stream of hydrogen through the blood for 2 hr. at 0° raised the pH to 7·80. Bishop (1923) showed in a similar manner that the pH of bee larva blood could be increased from 6·83 to 7·4, and he concluded that since there was no subsequent decrease in the pH, lactic-acid formation did not occur in bee-larva blood. An increase in the pH may also be obtained by dialysing the blood, the final pH being very close to that obtained in the above experiment with hydrogen. It will be shown below that this again is due to the loss of CO_2.

The blood of larvae which had been allowed to age for several days in a shallow dish of tap water tended to become more acid with time; but whether this was due to the formation of acidic substances by the insects, or to the action of bacteria with which larvae kept *in vitro* gradually become infected, is as yet undecided.

The pH of the larval blood measured at room temperature is no indication of what the physiological pH may be at the temperature of the insect's normal environment, which is some 20° C. higher. Accordingly, the pH of the blood was measured at 38° C., taking due precautions to keep the glass electrode, the larvae and the blood at this temperature during all stages of the measurement. The pH of a pooled blood sample at 38° C. was 6·64; the pH of another portion of the same sample measured at 14° C. was 6·80. Hence the pH-temperature coefficient for the

blood is –0·007 per 1° C. rise in temperature. The same value was reported by Dill, Daly & Forbes (1937) for mammalian serum at pH 6·6.

(*b*) *Carbon dioxide content.* The total CO_2 content of *Gastrophilus* blood drawn without exposure to air was found to be extremely variable; the average value from fourteen different batches of larvae was 72·4 vol. %, with a standard deviation of ±27·5 vol. % and extreme values ranging from 40·6 to 131·4 vol. %. To investigate whether the temperature of the larvae after removal from their host could in any way account for this variation, analyses were made of the CO_2 content of the blood from larvae collected on ice, and results were obtained similar to those found when larvae were collected at 38°. The temperature of the larvae could not, therefore, account for this variation, the most likely explanation for which will be discussed below.

The necessity for measuring the CO_2 content of the blood as soon as possible after the removal of the larvae from the horse is shown in the following experiment. The CO_2 content of the blood of a batch of larvae collected at 38° C. was measured 30 min. after removal from their host, a pooled blood sample from a large number of larvae being used to eliminate variations. The value so found was 64·5 vol. %. The remainder of the batch was allowed to remain in water at 38° C. for 3 hr., after which time the CO_2 content of the blood had decreased to 46·1 vol. %. If the experiment were repeated, but with the larvae kept on ice from the time that they were collected, no such decrease was observed. This may be explained by the observation that the larvae at 38° C. are very active and in continuous movement, whereas at 0° they are completely quiescent. It is known that lactic acid is liberated into the blood following muscular exertion during activity, and a certain extra amount of free CO_2 would be formed as a result, which could be easily lost by diffusion through the tracheal system. This may also partly account for the reason why the spiracles are widely opened during activity, even though the larvae are submerged in water. Since little or no lactic acid is formed at 0° and the spiracles are in the main kept closed, loss of CO_2 from the blood is greatly reduced. It may be noted that in the provision of an efficient spiracular closing mechanism, *Gastrophilus* exhibits an unusual feature among Dipterous larvae, and one which appears to be associated with an aquatic or semi-aquatic habitat.

Mammalian blood, if exposed to a vacuum, rapidly gives off the whole of its contained CO_2. A bicarbonate solution under similar conditions dissociates slowly to give off only half of its CO_2 according to the equation $2NaHCO_3 \rightleftharpoons Na_2CO_3 + CO_2$; blood plasma is intermediate between the two, and slowly gives off the bulk, *c.* 95%, of its total CO_2. To liberate the whole of the CO_2 from bicarbonate or plasma the addition of weak acid is required, the function of which is to accept the base liberated by the decomposition of the bicarbonate since the latter is unable to exist under zero CO_2 tension. In whole blood it is principally the haemoglobin which acts as the weak acid and accepts the liberated base; the plasma proteins, being both weaker acids and in lower concentration are much less efficient in this respect.

If *Gastrophilus* blood is shaken under vacuum in the Van Slyke apparatus with distilled water instead of the usual lactic acid, about 31 % of the total CO_2 is released. By expelling the CO_2 and repeating the operation three or four times, further

amounts of the gas are obtained until no more is given off. The remaining 5–7 vol. % of CO_2 can be released by treatment with acid in the usual manner. If the blood is evacuated by means of a good water pump for about 10 min. with frequent shaking, only 2–3 vol. % CO_2 remain when estimated in the Van Slyke apparatus. From the shape of the dissociation curve presented below it will be seen that at zero CO_2 tension the blood gives up its CO_2 completely; the small amounts remaining in the blood following evacuation are therefore probably due to either insufficient vacuum or too short a time of exposure. *Gastrophilus* blood, while apparently capable of giving up its entire CO_2 *in vacuo*, does so with considerably greater difficulty than is the case with mammalian blood. This may be explained, at least in part, by the fact that removing the bulk of the CO_2 raises the pH to a point where the CO_2 is more tenaciously retained, possibly in the form of carbonate.

The whole of the CO_2 of *Gastrophilus* blood is bound in a diffusible form, which may be demonstrated as follows. A sample of blood in a cellophane sac was dialysed against an isotonic (0·25 M) sodium chloride solution for 24 hr.; the total CO_2 was analysed before and after dialysis, and the result is shown below:

	Vol. %
CO_2 content of blood before dialysis	55·3
CO_2 content of blood after dialysis	2·4
CO_2 content of the external fluid	2·2

It can be concluded that the CO_2 is either not at all, or very loosely bound to the blood proteins. This observation is in marked contrast with mammalian blood, where only a part of the total CO_2 is dialysable.

Table 1. *Comparison of the* CO_2 *content of blood and tissues of* Gastrophilus

Exp. no.		CO_2 content in vol. %
1	Blood	55·0
	Tissues	50·1
2	Blood	98·4
	Tissues	94·4
3	Blood	64·5
	Tissues	56·1

It was of interest to determine whether the variations in the CO_2 content of the blood were in any way reflected by changes in the CO_2 content of the rest of the insect. The same larvae were used for the analyses on the blood and tissues, and the results are shown in Table 1. These figures show that carbon dioxide is very nearly equally distributed between the blood and tissues of the insect, and that any variation in the CO_2 content of the blood is accompanied by a similar change in the tissues. It cannot be inferred from this that the form in which the CO_2 is bound is necessarily the same, and there is in fact evidence from the literature to the contrary. Thus, Keilin (1921) showed that calcium carbonate in the form of 'calcospherites' may be found in quantity in the tissues of some Dipterous larvae, and it is almost

certain that the white amorphous substance in the Malpighian tubules of the *Gastrophilus* larva is this material. Other workers (e.g. Wallace & Hastings, 1942; Gesell & Hertzman, 1926) have found that the intracellular total CO_2 content was considerably lower than in the extracellular fluid, and have concluded that the cell membrane is impermeable to the bicarbonate ion. This conclusion was criticized by Conway & Fearon (1944), who showed that a considerable proportion of the total CO_2 in the mammalian muscle cell was bound in some unknown manner, i.e. Y-bound, and that the unequal distribution of bicarbonate between tissue cells and the surrounding fluid might be explained on the grounds of a Donnan equilibrium.

For purposes of comparison, a few determinations of the CO_2 content of the blood of early *Gastrophilus* pupae were made. The pupae were obtained by placing larvae during June-July in a dish with 1–2 in. of damp sawdust and keeping in an incubator at 24° C. The larvae commenced to pupae after approximately 48 hr., and this process was continued for about 4–5 days. The following values for the CO_2 content were obtained:

Age of pupa in days ...	2	4	6
CO_2 content of blood in vol. %	33·7	28·4	17·2

From these values it is seen that the earliest pupa has the highest CO_2 content in the blood, the value progressively decreasing as pupation proceeds. At 24° C. the pupal period is from 21 to 28 days, but no analyses were made after the sixth day on account of the difficulty in obtaining a fluid comparable to the original blood of the larva. By the fourth day already, the blood contains large numbers of amoebocytes, cell debris, etc., and had to be lightly centrifuged to remove these constituents, and subsequently it becomes thick and creamy. The blood of pupating holometabolic insects becomes dehydrated during pupation, and accordingly the figures shown above would be considerably lower if referred to the blood dry weight.

As has already been shown, the high CO_2 content of the larval blood falls after the larvae have been removed from the horse's stomach for any length of time. It is therefore most improbable that the high CO_2 content of the early pupa is due to CO_2 carried over from the larva; the most likely explanation is that the pupa is suffering from a partial asphyxiation due to its own endogenous respiratory CO_2, the liberation of which is considerably hindered by the formation of pupal cuticle and the less efficient functioning of the tracheal system. As pupation proceeds the intensity of CO_2 production declines—the respiratory metabolism of all insect pupae follows a U-shaped curve—and the pH tends to become more acid (e.g. see Agrell, 1948) with the result that the CO_2 content of the blood of older pupae would be low. Figures (and an explanation) similar to the above have been given by Courtois (1935) for pupae of the moth *Attacus polyphemus*. At the onset of pupation the pupa contains 28 vol. % CO_2 in the blood, which progressively declines as pupation proceeds; the blood of the larva contains only 9·0 vol. % CO_2.

(*c*) *The* pK_1' *of carbonic acid in the blood.* It is not immediately apparent that in the derivation of the pK_1' for *Gastrophilus* blood from equation (2) a knowledge of the Bunsen solubility coefficient α for CO_2 in the blood is required—see equation (5).

This has never been measured for the blood of any insect, but its approximate value may be deduced from a formula given by Danielson, Chu & Hastings (1939), viz.

$$\alpha_t^0 = 0{\cdot}530 + [0{\cdot}2 \times (\text{protein})] + [0{\cdot}0125 \times (38 - t)], \quad (4)$$

where α_t^0 is the solubility coefficient of CO_2 dissolved in 1 g. water of the fluid, at the temperature t, 0·530 is the solubility of CO_2 expressed in ml. per g. H_2O in 0·16 M-NaCl (i.e. a solution isotonic with mammalian blood, (protein) is the protein concentration of the fluid, expressed in g. per g. H_2O.

The solubility term then appears in the equation from which the total free CO_2 is, by definition, calculated as

$$[H_2CO_3] = \frac{pCO_2 \times 1000 \times \alpha_t^0}{760 \times 22{\cdot}26}, \quad (5)$$

where $[H_2CO_3]$ is expressed in mM. per kg. water and pCO_2 = the partial pressure of CO_2 in the gas phase.

Both Van Slyke, Sendroy, Hastings & Neill (1928) and Hastings *et al.* (1928) have pointed out that, for mammalian blood, an error of several per cent in the value for α would have an almost negligible effect on the calculated value for pK_1'. This is partly due to the fact that the value for $[BHCO_3]$ is obtained as the difference between the total $[CO_2]$ and $[H_2CO_3]$; in mammalian blood the ratio of $[BHCO_3]$ to $[H_2CO_3]$ is about 20:1, with the result that changes in α have a correspondingly small effect on $[BHCO_3]$. However, it will be shown below that in *Gastrophilus* blood the ratio of $[BHCO_3]:[H_2CO_3]$ may be in the region of 1, and for this reason the value of α has a much greater effect in calculating the pK_1' than is the case for mammalian blood.

The following determinations for the application of equation (4) have been made on *Gastrophilus* blood (Levenbook, 1950). The osmotic pressure is equivalent to a 0·25 M-NaCl solution, the protein concentration is 107·5 g./l. of blood, the water content is 84·3 % and the relative density 1·062. The fact that the salt concentration is higher than that for mammalian blood necessitates a small alteration in the first term of equation (4). From the paper of Van Slyke *et al.* (1928) it may be seen that the solubility of CO_2 in 0·23 M-NaCl is *c.* 0·52, and this value has therefore been used. The method of working out the pK_1' from a typical experiment is then as follows.

A sample of blood was equilibrated at 38° C. with CO_2 at a tension of 37·4 mm. Hg following which it had a CO_2 content of 22·4 vol. % and a pH of 7·09. From these data a value for pK_1' could now be calculated:

$$\text{Protein content in g./g. } H_2O = \frac{0{\cdot}1075}{1{\cdot}062 \times 0{\cdot}843} = 0{\cdot}1201,$$

$$\alpha_{38}^0 \text{ (from equation (4))} = 0{\cdot}52 + [0{\cdot}2 \times 0{\cdot}1201] = 0{\cdot}544,$$

$$[CO_2] \text{ in mM./kg. } H_2O = \frac{22{\cdot}4}{2{\cdot}226 \times 1{\cdot}062 \times 0{\cdot}843} = 11{\cdot}2,$$

$$[H_2CO_3] \text{ in mM./kg. } H_2O = \frac{37{\cdot}4 \times 1000 \times 0{\cdot}544}{760 \times 22{\cdot}26} = 1{\cdot}20,$$

$$[BHCO_3] \text{ in mM./kg. } H_2O = [CO_2] - [H_2CO_3] = (11{\cdot}2) - (1{\cdot}20) = 10{\cdot}00,$$

$$pK_1' = 7{\cdot}09 + \log \frac{1{\cdot}20}{10{\cdot}00} = 6{\cdot}17.$$

The mean value for the pK_1' of *Gastrophilus* blood at 38° C. and the experimental data on which the result is based are given in Table 2. The average value for the pK_1' of 6·08 with a standard deviation of ±0·06 is in close agreement with the corresponding figure of 6·10 at 38° C. found for mammalian blood by several workers (e.g. Dill *et al.* 1937; Hastings *et al.* 1928) and the 6·07 found by Bishop (1923) for bee-larva blood. Bishop considered that the correct value for bee blood should have been 6·10, i.e. the same as for mammalian blood. However, Hastings & Sendroy (1925) have shown that the pK_1' is depressed on increasing the ionic concentration; since this is higher in *Gastrophilus* blood than in mammalian blood it is very probable that the experimentally determined pK_1' for *Gastrophilus* really is slightly less than 6·10. It may also be seen from Table 2 that neither CO_2 tension nor CO_2 content of the blood appears to appreciably affect the pK_1'.

Table 2. *The* pK_1' *value for* Gastrophilus *blood at* 38° C.

Exp. no.	CO_2 tension in mm. Hg in gas phase	CO_2 content of blood in mM./kg. H_2O	pH	pK_1'
1	8·11	3·91	7·21	6·06
2	5·75	1·67	7·00	6·07
3	205	30·7	6·61	6·06
4	37·4	11·2	7·09	6·17
5	147·5	29·1	6·74	6·03
6	81·5	18·7	6·80	6·01
7	232	30·8	6·65	6·16
8	74·7	18·7	6·88	6·12
			Average	6·08

A number of measurements similar to the above were made at 16° C. The formula of Danielson *et al.* (1939) for α^0 is not correct at this lower temperature, as they assume a straight-line relationship between α^0 and temperature which does not hold in practice over a wide temperature range. For the present calculations a value for α^0_{16} of 0·987 was used, on the assumption that temperature has the same effect on α^0_t as on the solubility coefficient of CO_2 dissolved in pure water. The value for pK_1' so obtained from six experiments was 6·19 (S.D. ±0·13). This increase in the pK_1' as the result of a lower temperature has been observed for mammalian blood by Cullen, Keeler & Robinson (1925) and by Dill *et al.* (1937), and follows from the fact that decreasing the temperature decreases the dissociation constant for carbonic acid. At the same time both $[H_2CO_3]$ and $[BHCO_3]$ increase in amount (see Peters & Van Slyke, 1932) the increase being greater in the case of the carbonic acid. The result of these changes in equilibria is an increase in the value of pK_1'.

It was considered of interest to determine whether *Gastrophilus* blood could bind CO_2 in a form other than bicarbonate. Further experiments were therefore directed towards finding evidence for the formation of such compounds, with particular reference to carbamino formation, since insect blood contains a high concentration of free amino-acids (see Wigglesworth, 1939) which, especially at lower temperatures, could conceivably form carbamino compounds.

(*d*) *Kinetic measurements on* CO_2 *uptake and output.** Meldrum & Roughton (1934) demonstrated the formation of carbamate in mammalian blood by comparing the rates of CO_2 uptake by phosphate buffer, by blood to which cyanide had been added to inhibit the carbonic anhydrase and by normal blood. In each case there was an initial rapid uptake of CO_2 from the gas phase which, for normal blood, accounted for the whole of the CO_2 taken up. With phosphate buffer and cyanide-treated blood the rapid phase was succeeded by a prolonged slower rate of uptake, which was approximately linear with respect to time. By extrapolating the linear portions of the graph back to zero time, these results were explained as follows.

The initial rapid uptake by phosphate buffer alone was due to gaseous CO_2 going into physical solution, followed by the much slower hydration of further CO_2 to form bicarbonate. In cyanide-treated blood there was superimposed on the above a further rapid uptake of CO_2 due to carbamate formation, which resulted in a larger intercept on the ordinate at zero time. The distance between these two intercepts was a measure of the carbamino formation. With normal blood the now rapid hydration of CO_2 to bicarbonate (in the presence of carbonic anhydrase) was added to the two previous rapid reactions, and all the CO_2 to be absorbed, therefore, was taken up in one rapid reaction. The distance between the second and third intercepts was a measure of the bicarbonate formed.

Carbonic anhydrase is generally absent from the blood of insects (Florkin, 1935; Kreps & Chenykaeva, 1942); this has been confirmed for *Gastrophilus* blood and is demonstrated in Fig. 1. The lower dotted curve shows the rate of evolution of CO_2 from 2ml. of blood at pH 6·8, the upper dotted curve shows the greatly increased rate of CO_2 evolution from a similar sample to which 250 Meldrum-Roughton units of a crude preparation of horse carbonic anhydrase had been added. Further, the rate at which gaseous CO_2 was liberated from a bicarbonate solution in the boat was unaffected by the addition of *Gastrophilus* blood.

A typical experiment, based on the lines of those by Meldrum & Roughton described above, is shown by the top three curves of Fig. 1. The cyanide-treated blood of these authors corresponds to normal *Gastrophilus* blood, while mammalian blood would be equivalent to the insect blood with added carbonic anhydrase. It will be seen that the intercepts of curve 1 (CO_2 uptake by M/5 pH 6·8 phosphate buffer), and curve 2 (CO_2 uptake by normal *Gastrophilus* blood) at the ordinate are practically identical. On the addition of 250 Meldrum-Roughton units of horse carbonic anhydrase to the blood (curve 3), an amount of CO_2 equivalent to the height $A+B$ is rapidly absorbed in 150 sec., after which no more gas is taken up. Of the total CO_2 absorbed, the height A represents the fraction taken up in physical solution, the height B forming a measure of the bicarbonate fraction. The sum of $A+B$ accounts for the whole of the CO_2 absorbed with no fraction remaining unaccounted for. It is clear that under the conditions of this experiment carbamino formation does not occur. The same conclusion was derived from other similar experiments employing buffers of different pH and molarity, and the kinetic

* I am grateful to Dr A. M. Clark for his co-operation in these kinetic experiments.

measurements, therefore, produced no evidence for the formation of a CO_2 compound distinguishable from carbonic acid or bicarbonate.

It is of interest that by virtue of its high CO_2 capacity the same sample of *Gastrophilus* blood can be employed for measuring the rate of both output and uptake of CO_2, and that the final equilibrium point is the same. This may be seen on comparing curves 3 and 4 in Fig. 1.

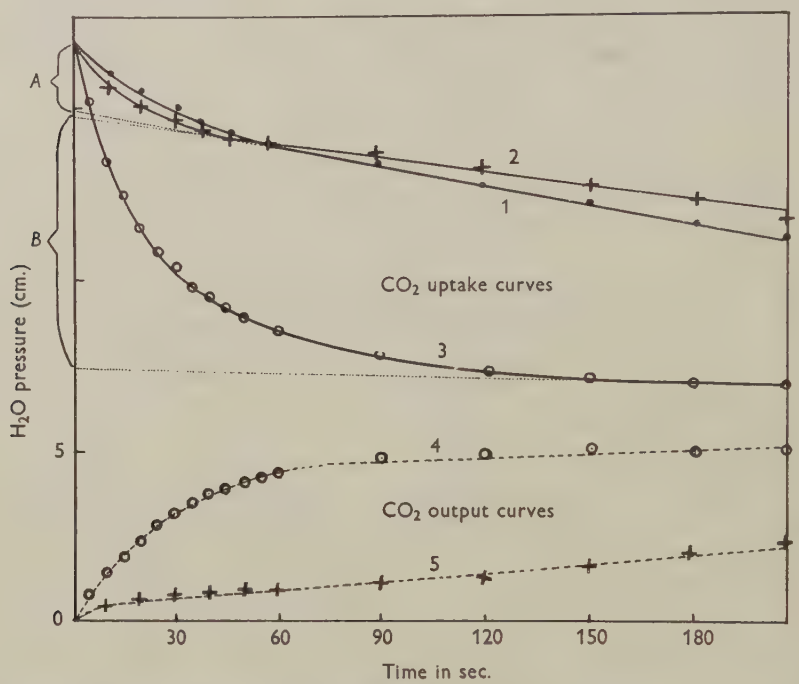

Fig. 1. Uptake and output of CO_2 by *Gastrophilus* blood. All curves refer to 2 ml. of blood, uptake curves + 2 ml. M/5 pH 7·2 phosphate buffer, output curves + 2 ml. M/5 pH 6·8 phosphate buffer. Temperature: 0° C. Curve 1, phosphate buffer; curve 2, normal *Gastrophilus* blood; curve 3, *Gastrophilus* blood + carbonic anhydrase. *A* and *B* represent the fractions of the total CO_2 absorbed in the forms of free CO_2 and bicarbonate respectively.

(*e*) *The* CO_2 *dissociation curve*. By equilibration of samples of *Gastrophilus* blood with different partial pressures of CO_2 as described above, the CO_2 dissociation curves at 38 and 16° C. were plotted, and these are shown in Fig. 2. Considerable variation existed in the shape of the dissociation curve from different samples of blood. The curve at 38° C. was drawn from two samples that had similar CO_2 binding properties, and was of the shape most frequently encountered. In the extreme case a curve was plotted from a blood sample which, at a tension of 80 mm. Hg contained 69 vol. % CO_2 instead of *c.* 37 vol. % for the more typical curve. Two samples were equilibrated at 16° C. and the corresponding curves are drawn dotted in Fig. 2; the mean of these two samples is drawn as the 16° C. curve.

The shape of the dissociation curve is somewhat different from that normally observed for other forms. The initial slope at low CO_2 tensions is rather gradual, and

in this respect is similar to that given by Bishop (1923) for bee-larva blood. At higher tensions, however, the slope does not fall off to the extent that might be expected by analogy with the gradual initial slope of the curves for the blood of other invertebrates (cf. Parsons & Parsons, 1923; Redfield, Coolidge & Hurd, 1926) *Gastrophilus* blood is therefore better adapted for functioning at higher CO_2 tensions. The curve also shows that at zero CO_2 tension the blood gives off its CO_2 completely,

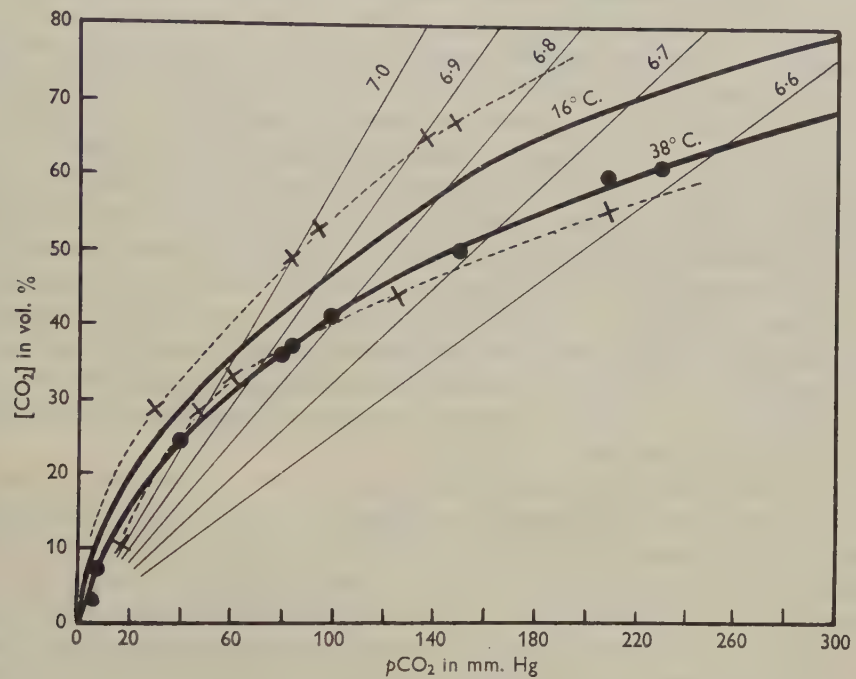

Fig. 2. The carbon dioxide dissociation curve for *Gastrophilus* blood. The iso-pH lines apply to the curve at 38° C. Dotted lines represent the limits of two curves plotted for blood at 16° C.

indicating that there is sufficient weak acid present to combine with all the base liberated from the bicarbonate. For the reasons discussed above, the blood at 16° contains more CO_2 than at 38°, and this is demonstrated by the difference in height of the curves at the two temperatures. This difference is not as large as that existing between the dissociation curves of other forms at different temperatures, e.g. sheep blood (Adair, Cordero & Shen, 1929), or skate blood (Dill, Edwards & Florkin, 1932), but it should be stressed that the 16° C. curve was computed from two widely varying blood samples, the mean of which may have given an artificially low curve.

DISCUSSION

It is of interest to compare the foregoing results with the conditions existing in the insect's environment, i.e. the stomach of the horse. Tappeiner (1883) gives the following figures for the gas composition in the horse's stomach: CO_2, 75%; O_2, 0·1%; CH_4, 0%; H_2, 14%; N_2, 10%. Although it undoubtedly varies at different periods, the gas in the stomach is approximately at atmospheric pressure, and hence from Tappeiner's data the CO_2 tension would be of the order of 500 mm. Hg. The CO_2 tension in equilibrium with the average 72·4 vol. % CO_2 content of *Gastrophilus* blood may be read off from the CO_2 dissociation curve at 38° C. as *c.* 300 mm. Hg, while from the Hasselbalch equation, assuming the same CO_2 content, a pK_1' of 6·10 and a pH of 6·40, the calculated tension is 335 mm. Hg. For a sample of blood with a CO_2 content of 120 vol. %—a not unusual value, the corresponding CO_2 tension would be 560 mm. Hg. This extremely high tension of CO_2 in the blood is certainly the result of the equally high tension existing in the stomach of the horse.

As a result of the high CO_2 tension in the blood, the amount of CO_2 carried in solution (i.e. H_2CO_3) is correspondingly large. Of the total CO_2 content of the blood, some 30–70%, depending upon the tension, may be accounted for as free CO_2 and the remainder as bicarbonate. For mammalian blood containing 50–60 vol. % CO_2, only about 5% is carried in solution, bicarbonate and to a lesser extent carbamate accounting for the rest.

The variation in the CO_2 content of the blood as drawn is certainly greater than could be expected from random sampling variations. The most obvious explanation is that it forms a measure of the variation in CO_2 tension in the stomach due to differences in the physiological state of the horse, and this is borne out by the observation that the variation in the CO_2 content amongst larvae taken from any one host is much smaller than that existing between larvae taken from different hosts. In this connexion also, an observation was made after these experiments had been completed which is of some interest, namely, that *Gastrophilus* larvae under the influence of bright illumination rapidly give off CO_2. Since this factor was not controlled in the present work, it may account for some of the variation observed.

Redfield, Humphreys & Ingalls (1929) showed that the differences that they observed in the shape of the CO_2 dissociation curves for the blood of the king crab *Limulus* were due to variations in nitrogen content of different samples of blood. They showed the nitrogen content was largely a measure of the haemocyanin present, and since this pigment was the principal blood buffer, this adequately accounted for the observed differences. A considerable variation in the protein nitrogen content of *Gastrophilus* blood has also been observed (Levenbook, unpublished), and it may well be that the variability in the shape of the dissociation curves is due to variation in the amount of blood protein, together with such substances as succinic acid (Levenbook & Wang, 1948), which could materially affect the amount of CO_2 bound.

SUMMARY

1. The pH of the blood of the third instar *Gastrophilus* larva is 6·64 at 38° C. with a pH-temperature coefficient of −0·007 per 1° C. rise in temperature.

2. The total CO_2 content of the blood varies from 40·6 to 131·4 vol. % with an average of 72·4 vol. %. The CO_2 content of the tissues minus the cuticle is very close to, and follows the variations in, the CO_2 content of the blood.

3. The CO_2 tension in the blood is from 300 to 500 mm. Hg. From 30 to 50% of the CO_2 is in solution, the rest in the form of bicarbonate. Carbamate formation does not occur in the blood.

4. The 'apparent' dissociation constant for carbonic acid, (pK_1'), has a value of 6·08 (S.D. ± 0·06) at 38° C. and 6·19 (S.D. ± 0·13) at 16° C.

5. CO_2 dissociation curves have been drawn for 38 and 16° C. The slope of the curves indicates that the whole of the CO_2 is given off at zero CO_2 tension, and that the blood is adapted for functioning at high CO_2 tensions.

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THE PHYSIOLOGY OF CARBON DIOXIDE TRANSPORT IN INSECT BLOOD

PART II. THE EFFECT OF INSECT BLOOD ON THE RATE OF HYDRATION OF CO_2

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(With Six Text-figures)

In a previous paper (Levenbook, 1950) it has been demonstrated that carbon dioxide transport in the blood of the larva of *Gastrophilus intestinalis* de Geer (Diptera) occurs largely in the form of bicarbonate. It is highly probable that this also applies to most other insects. The quantity of bicarbonate present is a function of both the available base and the carbonic acid concentration, the latter in turn being directly proportional to the amount of gaseous CO_2 existing in the blood. Wigglesworth (1939) has pointed out that except where the tracheolar endings are intracellular, the final gas transport between the tracheoles and the respiring tissues is through the insect's blood. Accordingly it is of interest to determine whether or not insect blood possesses any special properties peculiar to this function of transporting carbon dioxide between the cells and the tracheoles.

Kreps & Chenykaeva (1942, 1942*a*) studied the effect of Orthopteran blood on the rate of the reaction $H_2O + CO_2 \rightleftharpoons H_2CO_3$. The blood did not show any carbonic anhydrase activity, but they claimed that a substance was present which inhibited the physical hydration of CO_2 to form carbonic acid without having any effect on the reverse dehydration reaction. This substance was present in the cell-free plasma as well as in the deproteinized blood filtrate, but was absent from the tissues. It was thermostable, insensitive to cyanide, but was destroyed by ashing. They concluded from their studies that this substance—which was clearly no enzyme—was present in the blood of insects generally, its function being to ensure that respiratory CO_2 was kept in the gaseous form. As a result, elimination of the gas by diffusion through the tracheal system would be greatly facilitated; insect blood, therefore, possessed unique properties specially adapted for tracheal respiration. The present study was undertaken to confirm, and if possible extend, the work of the above authors.

MATERIAL AND METHODS

The insect blood employed for these experiments was obtained from two widely differing species of insects, third instar *G. intestinalis* larvae, and *Locusta migratoria migratorioides* adults. Haemolymph was obtained from the former in the manner

* 1851 Exhibition Scholar from Australia, 1947–9.

already described (Levenbook, 1950), while in the latter a coxa was amputated and the droplets of blood emerging on gently squeezing the body aspirated by means of a fine pipette.

Many methods, devised principally for the estimation of carbonic anhydrase activity, are available for measurement of the rate of the reaction

$$H_2O + CO_2 \rightleftharpoons H_2CO_3.$$

For the present purpose they may be divided into (*a*) colorimetric pH methods, and (*b*) manometric methods.

(*a*) *Colorimetric methods*

Colorimetric methods depend upon the principle that formation of H_2CO_3 and its subsequent ionization results in an increase in the hydrogen-ion concentration; hence, commencing with an alkaline solution, the time taken to attain an arbitrary more acid pH, as measured by the colour change of a suitable pH indicator, may be used as a measure of the rate of the above reaction. Three such methods were employed, using bromthymol blue and phenol red as indicators: (i) that originally due to Brinkman (1933), as subsequently modified by Kreps & Chenykaeva (1942*a*); (ii) the Philpot & Philpot (1936) method; (iii) an unpublished method recently devised by Dr D. D. Perrin of the Department of Colloid Science, Cambridge, which is briefly as follows.

Employing a modified Chance (1940) apparatus, a series of buffers of known pH containing a pH indicator is run through a capillary tube placed between a light source and a photocell. The changes in photocell current due to differences in light absorption are amplified and recorded by a pen-recorder. Having calibrated the apparatus in this manner, equal volumes of a saturated aqueous CO_2 solution and a suitable buffer containing the indicator are rapidly mixed in the apparatus. The flow of liquid is suddenly stopped, and as a result of hydration of CO_2 colour changes are recorded which, using the previous calibration curve, can be converted directly to give a pH-time curve. Provided that the pH range covered is approximately 6·4–8·4 and that the pK of the buffer is near 7, these results are capable of exact mathematical treatment to give a value for the first order velocity constant for the rate of hydration of CO_2. The influence on the velocity constant of any substance added to the buffer may therefore be investigated. Allowances can be made for any extraneous effects by including the same substance in the buffers used for the original calibration curves.

(*b*) *Manometric methods*

For manometric measurements of the rate of the hydration reaction, the technique was similar to that already described (Levenbook, 1950), the only difference being that the temperature of the water-bath was 15° instead of 0° C. The use of the higher temperature ensured that the non-enzymatically catalysed rate of hydration of carbon dioxide was sufficient to enable any inhibitory effects to be readily observed.

RESULTS

(i) The effect of insect blood on the rate of hydration of CO_2, as measured by the Brinkman technique, is summarized in Table 1.

Table 1. *The effect of insect blood on the rate of hydration of* CO_2 *as measured by the Brinkman technique*

	Time for change of indicator colour (sec.)	
	Phenol red	Bromthymol blue
Control	100	120
As above + 0·31 % (0·05 ml. 1:3 diluted) locust blood	180	122
Control	110	—
As above + 0·31 % (0·05 ml. 1:3 diluted) *Gastrophilus* blood	130	Values unobtainable, see text

It will be seen that, using phenol red, the addition of either locust or *Gastrophilus* blood brought about a considerable increase in the time required to change the colour of the indicator. (Such an increase in time will be referred to below as an 'inhibition', but it will be shown subsequently that this is not a true inhibition of the rate of the $CO_2 + H_2O$ reaction.) The 'inhibition' amounted to some 80% with locust blood, but only 18% for that of *Gastrophilus*. These figures generally confirm the observations of the Russian authors for Orthopteran blood; but very different results were obtained when bromthymol blue was used as the indicator. In this case the presence or absence of locust blood made very little difference to the apparent rate of reaction, while with *Gastrophilus* blood, on mixing the reagents, the indicator immediately changed from blue to a greenish shade and showed no appreciable further change during the next few minutes. For this reason it was impossible to attain an exact end-point.

The considerable difference between the 'control' times for the two indicators is probably connected with the slightly more acid pH—and hence the longer time necessary to attain this value—at which bromthymol blue changes colour (pH 6·0–7·6) as compared with phenol red (pH 6·8–8·4).

(ii) A set of typical results employing the Philpot & Philpot technique is given in table 2.

Using bromthymol blue, the usual indicator for this method, the effect of adding 0·25 ml. of 1:3 diluted blood of either locust or *Gastrophilus* was much the same, namely, a small 'inhibition' of the order of 10%; a similar result was obtained with phenol red (13%). In view of the low magnitude of these effects as compared with those obtained by the Brinkman method, the concentration of blood was increased to see whether this would result in a greater degree of 'inhibition'. In part this proved to be the case, since increasing the quantity of blood to 0·5 ml. approximately doubled the 'inhibition' previously obtained, whereas 1 ml. of blood was without further effect.

Table 2. *The effect of insect blood on the rate of hydration of* CO_2 *as measured by the Philpot & Philpot technique*

	Time for change of indicator colour (sec.)	
	Bromthymol blue	Phenol red
Control	38	38
As above + 0·55 % (0·25 ml. 1:3 diluted) locust blood	43	41
Control	38	38
As above + 0·55 % (0·25 ml. 1:3 diluted) *Gastrophilus* blood	50	42
As above + 4·5 % (0·5 ml. undiluted) *Gastrophilus* blood	63	52
As above + 9 % (1·0 ml. undiluted) *Gastrophilus* blood	64	54

(iii) In Figs. 1 and 2 are shown the combined data of a series of runs using the Perrin method with veronal buffer containing phenol red, and phosphate buffer with bromthymol blue, with and without the addition of *Gastrophilus* blood. When using bromthymol blue in the presence of *Gastrophilus* blood the sensitivity of the instrument was considerably reduced on account of the greenish colour already noted above; but this did not affect the final results as the blood was also included in the reference calibration curves employed for these experiments.

It will be seen that the slopes of the curves in the presence or absence of blood are identical within the limits of experimental error of the method, which demonstrates that *Gastrophilus* blood is without any effect on the velocity constant for the $H_2O + CO_2 \rightleftharpoons H_2CO_3$ reaction.

When the results of the experiments on the manometric measurement of the rate of hydration of CO_2 are plotted graphically, it is seen that there is an initial rapid uptake of CO_2, followed after about 30 sec. by a slower rate which is virtually linear with respect to time. The rapid uptake at the beginning is due to CO_2 going into physical solution together with any carbamate formation, and the shape of this portion of the graph is of no importance in the present work. The slope of the linear portion of the graph, however, is a direct measure of the rate of hydration of CO_2. Conclusive evidence for the presence of an inhibitory substance in insect blood would be obtained if, in the presence of the latter, there was a significant decrease in the slope of this curve.

Typical experiments of the above type using locust and *Gastrophilus* blood are shown in Figs. 3 and 4. Again it will be seen that when runs in either the presence or absence of insect blood are compared, there is no difference in the slope of the linear portion of the graphs. Altering either the pH or molarity of the phosphate buffer, or both, in no way altered this result. It may be concluded, therefore, that the blood of neither of these insects contains any substance which inhibits the

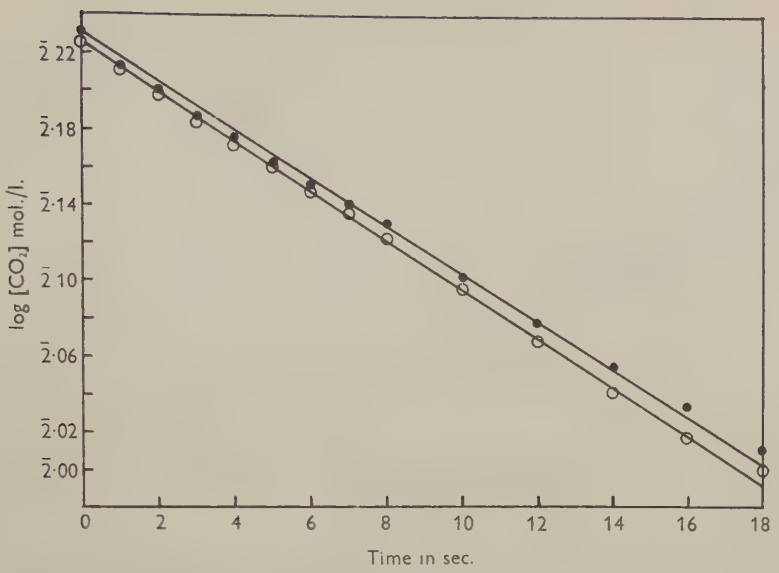

Fig. 1. The effect of *Gastrophilus* blood on the rate of hydration of CO_2 as measured by the Perrin method. The final solution contained: 0·001 % phenol red, 0·01 M pH 8·6 veronal buffer, 0·0172 M-CO_2. Circles: control solution, dots: the mean of three runs with a similar solution containing 2 % *Gastrophilus* blood. Temperature, 18·5° C.

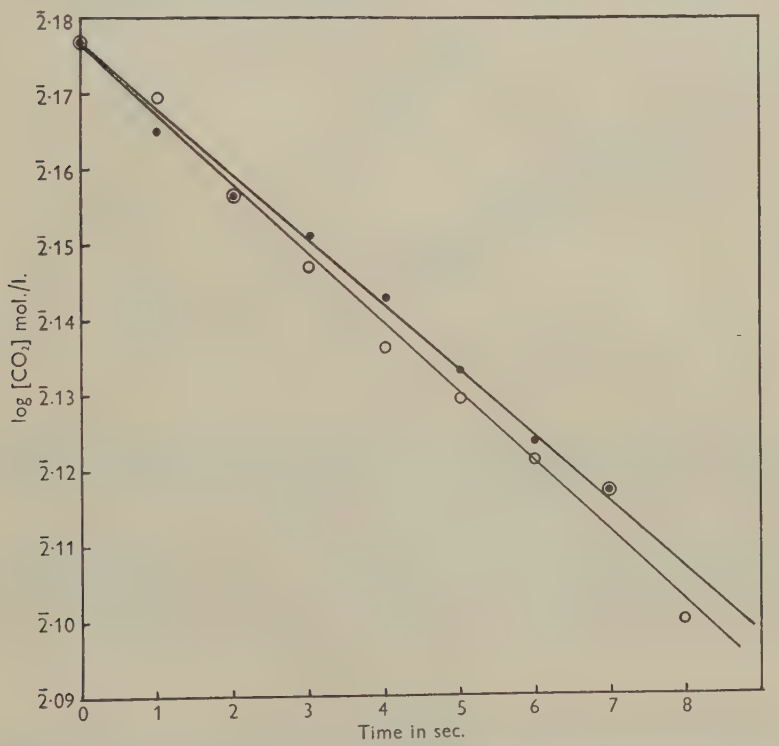

Fig. 2. The effect of *Gastrophilus* blood on the rate of hydration of CO_2 as measured by the Perrin method. The final solution contained: 0·001 % bromthymol blue, 0·01 M pH 8·0 phosphate buffer, 0·015 M-CO_2. Circles: control solution, dots: the mean of two runs with a similar solution containing 2 % *Gastrophilus* blood. Temperature, 20° C.

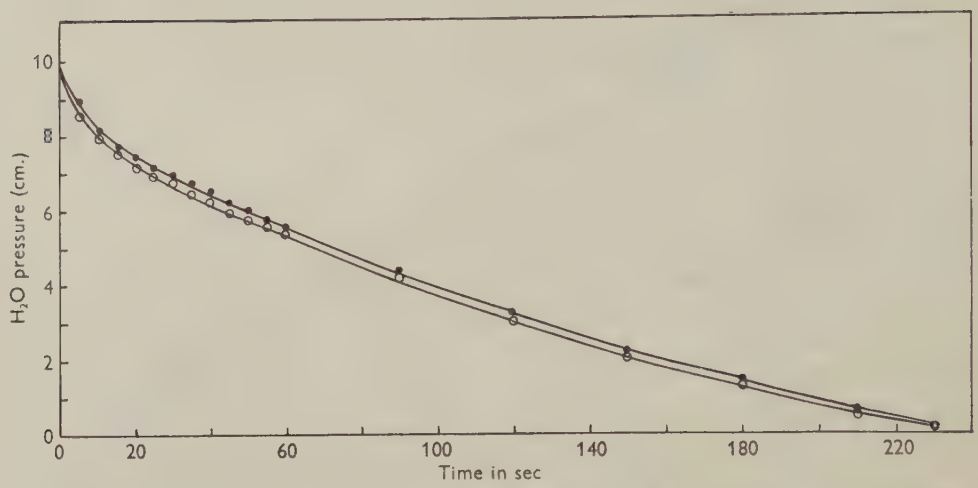

Fig. 3. The effect of locust blood on the rate of uptake of CO_2 as measured manometrically. The boat contained 4 ml. of 0·04 M pH 7·2 phosphate buffer. Dots: control, circles: a similar solution containing 0·4 ml. locust blood. Temperature, 15° C.

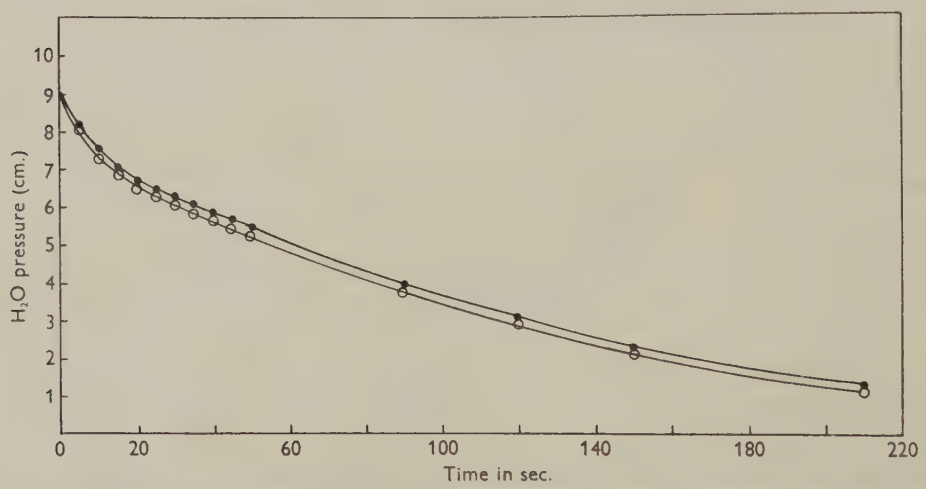

Fig. 4. The effect of *Gastrophilus* blood on the rate of uptake of CO_2 as measured manometrically. The boat contained 4 ml. 0·04 M pH 7·2 phosphate buffer. Dots: control, circles: a similar solution containing 0·4 ml. *Gastrophilus* blood. Temperature, 15° C.

hydration of carbon dioxide. It thus appears that the observed 'inhibition' obtained by the Brinkman and Philpot & Philpot methods is spurious, and must be attributed to some factor or factors inherent in these colorimetric methods.

DISCUSSION

From the data presented it is clear that, depending upon the technique employed, very different conclusions may be drawn regarding the effect of insect blood on the rate of hydration of carbon dioxide. Thus, with the manometric or Perrin colori-

metric methods, the blood is without influence on the rate of this reaction, whereas with the Brinkman or Philpot & Philpot techniques the rate appears to be inhibited to an extent dependent upon the conditions of the experiments. The results obtained by these latter procedures are largely vitiated, however, mainly for the following reasons.

(1) Danielli (1941) has pointed out that, with cationic indicators such as were used in the present experiments, the indicator error due to protein on the alkaline side of its isoelectric point may be very considerable; under these conditions the indicator measures the pH at the surface of the protein instead of the bulk phase. It might be anticipated therefore, that an indicator error would be manifested in the presence of proteins, free amino-acids, salts, etc., all of which are present in insect blood and possibly in other biological material.

A pronounced indicator error would account for the immediate change in colour of bromthymol blue observed on the addition of *Gastrophilus* blood. The magnitude of the error in this case may clearly be seen from Fig. 5; this shows the relation

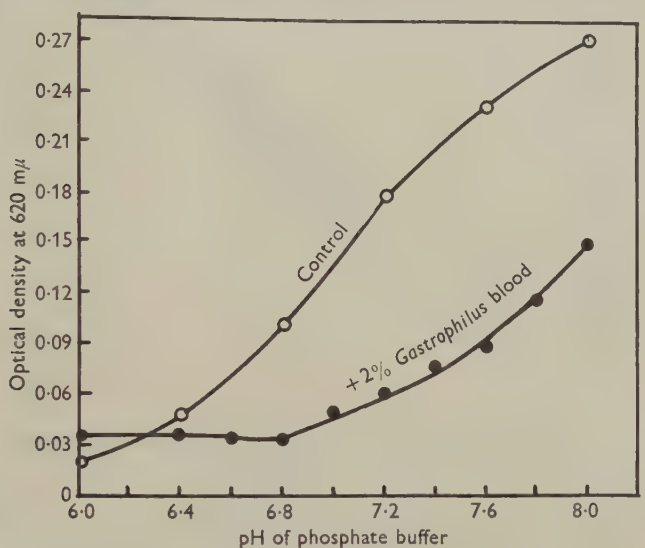

Fig. 5. The effect of *Gastrophilus* blood on the colour of bromthymol blue. The pH of the buffer + blood solutions were checked with the glass electrode.

between optical density and pH for a bromthymol blue solution as measured in the Beckman spectrophotometer at a wave-length of 620 mμ, which represents the point of maximum absorption at pH 8. Any method, therefore, involving colour matching of a solution of bromthymol blue cannot be accurate if only one of the solutions contains any material such as *Gastrophilus* blood.

Spectrophotometric measurements of buffered phenol red solutions, with and without *Gastrophilus* blood, showed that for this indicator the comparable protein error was considerably smaller, as may be seen from Fig. 6.

(2) In the Brinkman method the observed rate of the reaction is largely governed by the time it takes to convert the small amount of carbonate present to bicarbonate.

The magnitude of this quantity may be judged from the fact that in the original method (Brinkman, 1933) the only carbonate available is the small amount present as an impurity in the 0·02 M-$NaHCO_3$ solution used. Kreps & Chenykaeva (1942*a*) improved this method by making the final solution approximately 0·0002 M in carbonate, but even at this concentration the system is still very sensitive to traces of H^+ or OH^- introduced with the material under examination. Thus, using the Kreps & Chenykaeva procedure, 'inhibition' or 'activation' in the presence of *Gastrophilus* blood could be obtained at will depending upon the pH chosen for the final comparison, and these effects could be closely simulated by the addition of a similar amount of 0·005 M phosphate buffer of pH 7, which is approximately equal to that of *Gastrophilus* blood. Using a higher carbonate concentration—and hence increasing the poise of the carbonate-bicarbonate buffering system—the 'inhibition' due to the insect blood was no longer observed.

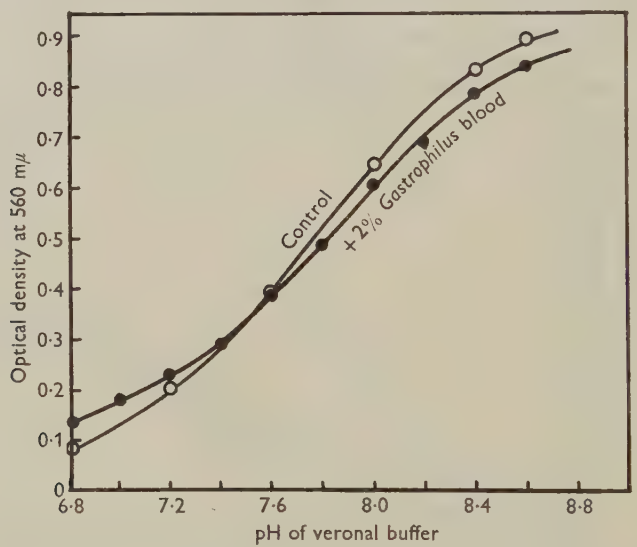

Fig. 6. The effect of *Gastrophilus* blood on the colour of phenol red. The pH of the buffer + blood solutions were checked with the glass electrode.

Regarding the Philpot & Philpot (1936) method, it should be pointed out that, although criticism has been directed against it both in the present paper and by Roughton (1943), it was never intended as a precise quantitative method, but simply as a bench laboratory demonstration of the effect of carbonic anhydrase.

SUMMARY

1. A manometric and an improved colorimetric method have been employed to study the effect of insect blood on the reaction between carbon dioxide and water.

2. The absence of carbonic anhydrase from insect blood has been confirmed.

3. Neither Locust nor *Gastrophilus* larva blood contains any substance which inhibits the hydration of carbon dioxide.

The authors would like to express their thanks to Prof. D. Keilin, F.R.S., for his interest in the present work, and to Dr D. D. Perrin for making the measurements involving the use of his apparatus and for valuable discussion.

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THE PHYSIOLOGY OF CARBON DIOXIDE TRANSPORT IN INSECT BLOOD

PART III. THE BUFFER CAPACITY OF *GASTROPHILUS* BLOOD

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(With Three Text-figures)

One of the primary attributes of the internal medium of any organism is that it should maintain its normal hydrogen-ion concentration within certain rather narrow limits. Throughout life and, for different reasons, even after death, there is a continuous tendency for this pH to be depressed on account of the liberation into the blood of certain predominantly acidic metabolites, together with respiratory carbon dioxide. Although insects appear to support a wider range of pH change than do mammals, if their blood were insufficiently well buffered, its reaction would soon become outside the limits compatible with life.

The resistance to alteration in pH, or the buffering capacity β, of a fluid has been defined by Van Slyke (1922) as 'the differential ratio dB/dpH, which expresses the relationship between the increment (in g. equivalents per litre) of strong base B added to a buffer solution and the resulting increment in pH. Increment of strong acid is equivalent to a negative increment of base or $-dB$. In these terms, therefore, a solution has a buffer value β, of 1, when a litre will take up 1 g. equivalent of strong acid or alkali per unit change of pH'.

The buffer capacity of the blood of a few insects has been previously investigated, and shows certain characteristic features. These insects include the larva of *Prodenia eridania* (Babers, 1941), *Pieris rapae* and *Heliothis armigera* (Craig & Clark, 1938), the adult Mormon cricket, *Anabrus simplex* (Pepper, Donaldson & Hastings, 1941), and various other Orthoptera (Hastings & Pepper, 1943). In general, the blood of insects differs from vertebrates in that, although the buffer capacity may vary widely from one insect to another, it is always lowest in the region of the normal pH of the blood, instead of being best buffered at this pH as is the case in most other animals. This means that the value for β increases on either side of the normal pH of the blood, so that the buffer value curves (i.e. a plot of β against pH) are U-shaped, whereas vertebrate blood buffers less efficiently on either side of its normal pH, and the buffer value curve is ∩-shaped. Further, the curve for insect blood is generally fairly smooth and shows few, if any, inflexions. This indicates that a whole series of overlapping buffer systems are involved, and no single one of them predominates. In animals which possess haemoglobin or haemocyanin, these respiratory pigments represent the dominant blood buffer.

In the blood of most terrestrial insects, the concentration of bicarbonate is generally

low. Now in the neutralization of a non-volatile acid or base, bicarbonate is of special and unique importance. Addition of acid to a solution containing bicarbonate, results in the formation of the neutral acid salt and liberation of an equivalent amount of CO_2; since this acid gas is volatile, the resulting excess can be rapidly removed from the blood through ventilation by the respiratory system. As long as any bicarbonate still remains therefore, there is relatively little change in the pH of the blood. Of course, if the invading acid is either carbonic acid itself or a weaker acid, this mechanism is of no avail.

In the blood of the larva of *Gastrophilus* it was demonstrated (Levenbook, 1950*a*) that the bicarbonate concentration is considerably higher than in the haemolymph of many other insects. Accordingly, it was of interest to determine the buffering power and the importance of the bicarbonate in this particular type of insect blood.

MATERIAL AND METHODS

Blood from mature third instar *Gastrophilus intestinalis* de Geer larvae was collected under paraffin oil as described in a previous paper (Levenbook, 1950*a*). The larvae were kept at 38° C., and were sacrificed within 1 hr. after the time they were removed from the horse's stomach.

When sufficient blood had been collected, 3 ml. were sucked up from under the paraffin and pipetted into a short wide tube of approximately 10 ml. capacity. Small additions of either N/10-HCl or NaOH were made from a 1 ml. burette with continuous mechanical stirring. After each addition of acid or soda, about 30 sec. was allowed for equilibration, and the pH measured with a glass electrode. For determinations where the pH was 8 or higher, a special alkali electrode standardized against M/20 borate buffer was employed. No special precautions were taken for temperature control, the room temperature being $20 \pm 1°$ C. during all the measurements.

Dialysed blood was obtained as follows: known volumes of blood were dialysed in a cellophane sac against a large volume of isotonic (0·25 M)-NaCl solution for 24 hr. at 0°. In all cases an increase in blood volume was obtained which, on account of the non-diffusible protein, was probably due to a Donnan effect. The whole sample was then subsequently titrated, and the results calculated back to the original blood volume.

Carbonic anhydrase was prepared from horse blood by the alcohol-chloroform method described by Meldrum & Roughton (1934). A known weight of the previously tested, dried enzyme powder was added to the blood as required, and a similar sample added to distilled water served as a control for the buffering power of the enzyme protein.

RESULTS

The results of composite titration curves from three samples of normal *Gastrophilus* blood, dialysed blood, and blood to which carbonic anhydrase had been added (the latter corrected for the buffering effect of the added enzyme), are shown in Fig. 1.

The buffer capacity of the blood is equal to the slope of the titration curves at any

particular pH (Van Slyke, 1922), and the buffer capacity curves shown in Fig. 2 were obtained by drawing tangents to the titration curves at suitably chosen pH intervals and measuring their slope.

Small inflexions in the titration curve can produce much larger effects on the shape of the buffer value curve if this is derived in the above manner. The latter curves were therefore checked by calculating a number of values for β directly—a theoretically accurate procedure providing dB and dpH are sufficiently small—and substantially identical curves were obtained by either method.

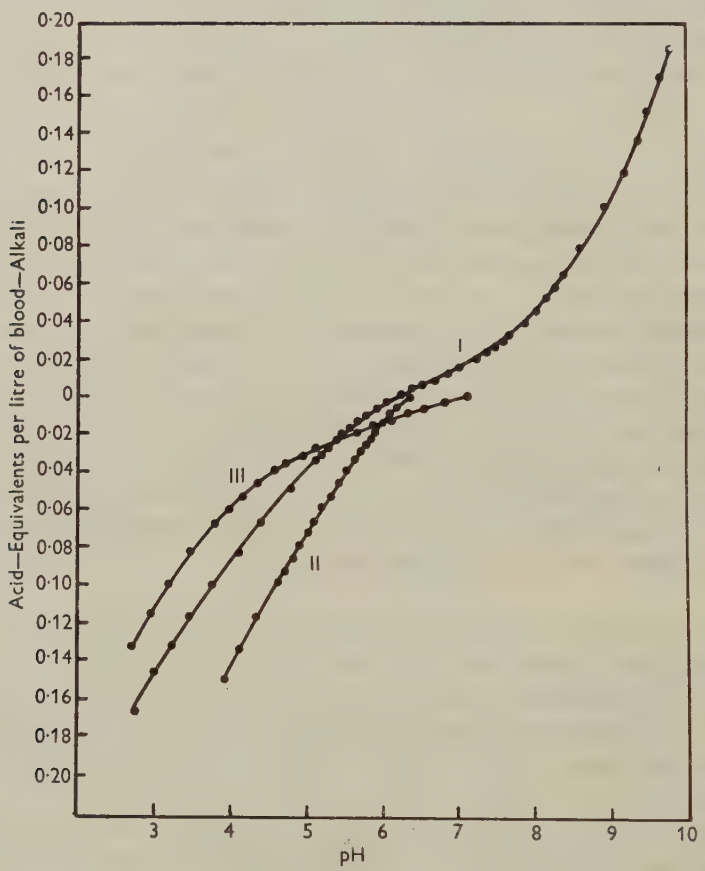

Fig. 1. Titration curves of *Gastrophilus* blood. I = normal blood; II = blood + 148 Meldrum-Roughton units of carbonic anhydrase; III = dialysed blood. Note the increased initial pH for the latter curve due to the loss of CO_2.

The buffer value curve for normal *Gastrophilus* blood is of the general type already described for the haemolymph of other insects. Its similarity to the curve for *Prodenia eridania* larva blood is particularly striking, even including the small inflexion at pH 6·8. This is all the more remarkable when the approximately 8-fold difference in the CO_2 content of the blood of these two insects is taken into account.

Babers (1941) considered the buffering due to bicarbonate was negligible in *Prodenia* blood, but this is certainly not the case for *Gastrophilus*.

The buffer value curve for the dialysed blood is due to the non-dialysable blood constituents—almost entirely proteins. At the average blood pH of 6·8, the β value for dialysed blood is 0·014, and for whole blood at the same pH, 0·0225. The proteins thus appear to account for some 62% of the total buffering power, bicarbonate and other diffusible buffers making up the remainder. This figure may be also derived in another manner.

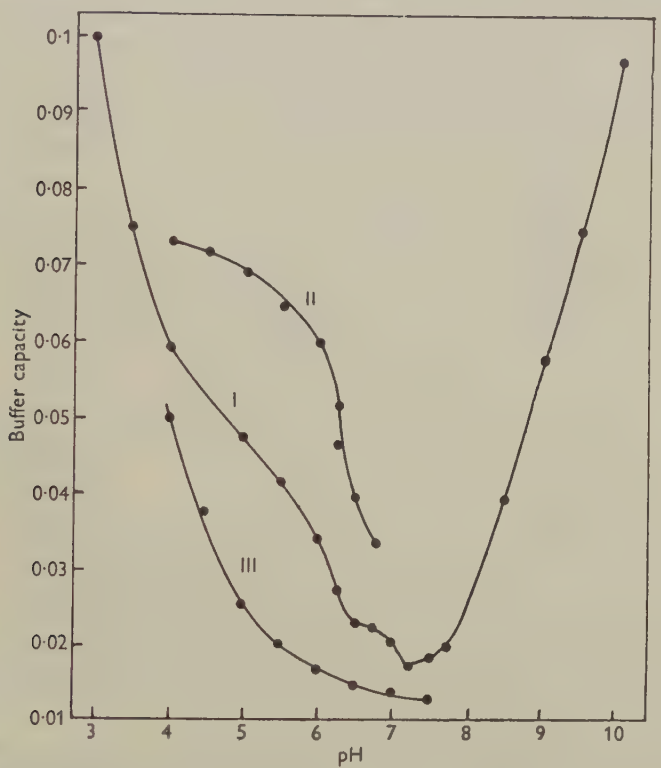

Fig. 2. Buffer value curves for *Gastrophilus* blood. I = normal blood; II = blood + 148 Meldrum-Roughton units of carbonic anhydrase; III = dialysed blood.

It has been pointed out by Van Slyke in a number of papers (e.g. Van Slyke, Hastings & Neill, 1922) that an increase in base bound as $BHCO_3$ is equivalent to the loss of base by other buffers of the blood, i.e. $\frac{dB}{d\text{pH}} = -\frac{d[BHCO_3]}{d\text{pH}}$. Now from measurements made to derive a value for the pK_1' of carbonic acid in *Gastrophilus* blood at 16° C. (Levenbook, 1950*a*), the bicarbonate concentration at any particular pH may be calculated, and plotted against the pH. This has been done in Fig. 3 and, as in the case of mammalian blood, over the limited pH range covered, the graph is virtually a straight line. The slope of this graph represents the equation given above and is a measure of the buffering capacity of substances other than

bicarbonate, and in the present instance equivalent to a buffer value of 0·0142 when [$BHCO_3$] is expressed in mol./l. This agreement with exactly the same value for the β of dialysed blood is undoubtedly coincidental, since although protein, apart from bicarbonate, accounts for the major portion of the buffering power, other less important buffering substances are also present.

These other substances which have been suggested as buffers in insect blood include phosphoric and uric acids (Babers, 1941) and the free amino-acids (Bishop, 1923). For *Gastrophilus* blood, succinic acid (Levenbook & Wang, 1948) must also be included, and its buffering effect may be evaluated as follows.

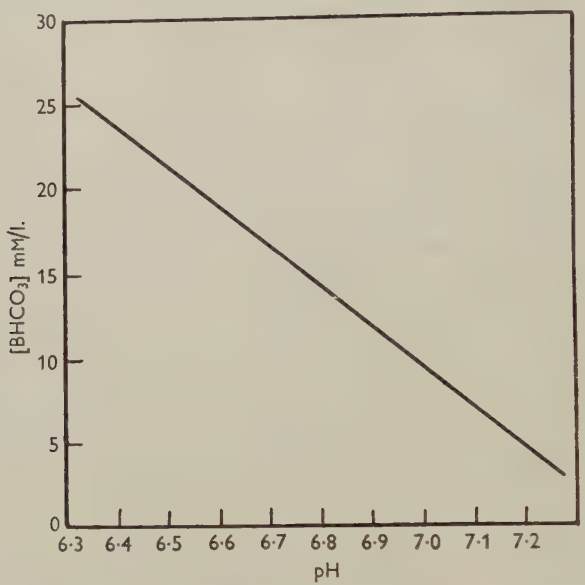

Fig. 3. pH-[Bicarbonate] curve for *Gastrophilus* blood at 16° C. [Bicarbonate] was calculated from the measured pH and CO_2 content, using the experimentally determined pK'_1 value of 6·19 (Levenbook, 1950*a*). At pH less than 6·2, the graph progressively departs from linearity.

Van Slyke (1922) has shown that the buffering of any acid or base is maximal when the pH is equal to the pK′ of the substance concerned, and moreover, for a monovalent acid, or a polybasic acid where the respective pK′ values are more than 2·5 units apart, the maximum molar buffer value is 0·575, i.e.

$$C = \frac{\beta}{0{\cdot}575},$$

where C is the molar concentration.

The succinate concentration in *Gastrophilus* blood is *c.* 0·0205 M (Levenbook & Wang, 1948), and the second pK′ of succinic acid is 5·55. The first pK′ is sufficiently below the second for this dibasic acid to conform to the conditions given by Van Slyke above. Substituting the pK′ value and the normal blood pH of 6·8 in the Henderson-Hasselbalch equation $pH = pK' + \log \frac{[salt]}{[acid]}$, it may be calculated that the ratio of salt to acid is 13 : 1. The buffering effect of this system therefore increases

as this ratio approaches 1, i.e. as the pH approaches 5·55, and at this point the β would be maximal, with a value of $(0{\cdot}0205 \times 0{\cdot}575) = 0{\cdot}012$. However, at pH 6·8 the buffering effect is only about one-fifth of this figure, of the order of 0·002.

Uric acid and the free amino-acids contribute a negligibly small fraction towards the total buffer value; Levenbook (1950*b*) found the former to be present in only very small amounts, and although the concentration of the latter had the high value of *c.* 0·5%, the pK values for the buffering groups of the amino-acids are so far from the normal pH of the blood, that it is extremely doubtful to what extent they could act as buffers in the physiological pH range.

At pH 6·8 the phosphates are buffering at their maximum efficiency. If the assumption is made that only the inorganic phosphate of the blood is involved, then it may be shown that the 0·003 M-phosphate in the blood has a buffer value of approximately 0·0017.

The above calculations show that of the total β value of 0·014 for buffers other than bicarbonate, succinate, phosphate and other minor buffering constituents probably account for a buffer capacity of close to 0·004, and therefore protein for approximately 0·01. The average protein content of *Gastrophilus* blood is 107·5 g/l. (Levenbook, 1950*b*), so that the buffer value per g. of protein is

$$0{\cdot}01/107{\cdot}5 = 9{\cdot}6 \times 10^{-5},$$

which is the same as that obtained for horse serum protein (Van Slyke, Hastings, Hiller & Sendroy, 1928), but considerably below the 13×10^{-5} for human haemoglobin (Adair, 1925).

he effect of carbonic anhydrase on the buffer value curve must now be discussed.

Brooks & Pace (1938) have pointed out that the buffering power of bicarbonate varies within the limits of two extreme conditions, viz. (*a*) in the absence of a gas phase, (*b*) in the presence of a gas phase with constant pressure of CO_2. Following the addition of a non-volatile acid or base, the bicarbonate is a much more effective buffer under the latter conditions, owing to the possibility of exchanging CO_2 with the gas phase. Thus in a solution containing both protein and bicarbonate, the relative fraction of the total buffering capacity contributed by the protein is smaller in case (*b*) than in case (*a*).

In the foregoing discussion, it has so far been assumed that during the titration of the insect blood, since the pH reading some 30 sec. after each addition of acid or alkali was steady, therefore equilibrium conditions had been reached with the constant CO_2 (i.e. atmospheric) gas phase. Now from Fig. 2 it will be seen that addition of carbonic anhydrase produced a considerable increase in the buffer capacity of *Gastrophilus* blood. This can only mean that equilibration between the CO_2 liberated from bicarbonate on acid titration and that in the atmosphere was in fact incomplete, and the prevailing conditions were therefore intermediate between cases (*a*) and (*b*) described above. After the addition of carbonic anhydrase, a true equilibrium was more rapidly attained, resulting in an increased buffering effect for the reason already given.

Under these conditions the value for β is not 0·023 as originally found, but some

50% higher, i.e. 0·033; as the contribution of buffers other than bicarbonate is still the same, bicarbonate now accounts for some 57·5% of the total buffering power, instead of *c.* 38% as found in the absence of carbonic anhydrase.

DISCUSSION

A priori it might have been expected that as in the case of most other animals, it would also be advantageous for insects if the optimum buffering power of their blood occurred at its normal pH. In point of fact it has been shown that this is not so, and the following tentative explanation may therefore be suggested for the significance of the U-shaped buffer value curves of insect blood.

It has already been pointed out above that lactic acid liberated into the blood following muscular exertion—and in organisms as active as insects the amount may be quite considerable (Bodine, 1928; Schütze, 1932)—increases the free CO_2 content of the blood at the expense of bicarbonate. Activity also causes an increase in the respiratory rate, which would further increase the CO_2 tension in the blood. Now, Lee (1925), Hazelhoff (1927), Wigglesworth (1935) and others, have shown that an increase in blood lactic acid, or a decreased pH due to an excess of CO_2, generally causes insects to open their spiracles and, with higher concentrations of CO_2, respiratory movements are induced for the purpose of ventilating the tracheal system. The physiological response to excess CO_2 in the blood by insects, therefore, is similar to that in vertebrates, namely, an endeavour to remove this excess by increasing the rate of diffusion of CO_2 through the respiratory system. However, in this respect the insect tracheal system is probably much less efficient than the vertebrate lung, for the following reasons.

The insect circulatory system is comparatively sluggish, and, as pointed out above, CO_2 diffuses only slowly out from the blood into the gas phase due to the absence of carbonic anhydrase. Further, ventilation in insects favours an increased supply of oxygen rather than the elimination of CO_2, even though the magnitude of this process may be relatively almost as great as in man (cf. Wigglesworth, 1931).

As a consequence of this insufficiency in tracheal respiration, the CO_2 content of the blood might reach such a high value that the increase in hydrogen-ion concentration could result in tissue damage; but by virtue of the fact that the blood is progressively better, instead of worse, buffered the greater its change in pH, such a situation is unlikely to arise, and the insect's further activity would not be impeded as the result of its own metabolism.

SUMMARY

1. From titration data it has been calculated that the buffer capacity β for normal *Gastrophilus* blood at pH 6·8 is 0·0225, for dialysed blood 0·014 and for blood to which carbonic anhydrase has been added, 0·033. The buffer capacity curves are all more or less U-shaped.

2. The contribution towards the total buffer capacity of various buffering substances, of which protein and bicarbonate are the most important, has been quantitatively determined.

3. It is tentatively suggested that the reason why insect blood is better buffered on either side of its normal pH instead of the reverse as in other animals, may be related to the inefficiency of the tracheal respiratory system in eliminating excess CO_2.

I should like to thank Prof. D. Keilin, F.R.S., for his interest and advice in the present work.

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FLIGHT RECORDERS. A TECHNIQUE FOR THE STUDY OF BIRD NAVIGATION

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(With Three Text-figures)

Experiments on the homing ability of birds usually take the form of displacing a bird from its home, releasing it at a known time, observing its behaviour until out of sight and then noting the time when it returns, if at all, to its home. If the interval between release and return is that appropriate to direct flight from release point to home the interpretation is obvious; the bird has flown more-or-less straight home. If, as is more usual, the interval is considerably greater than the direct flight time the question arises as to whether the bird has been flying all the time or whether some substantial fraction of the time has been spent in other ways such as resting and feeding. It is of importance to be able to answer this question: a theory of bird homing based on methodical or random search would demand a considerable time spent actually on the wing, while a theory which postulates some genuine navigational ability on the part of the bird would admit of considerable resting periods and a time actually in the air no bigger than the direct flight time. The time spent in flying is, then, a vital datum which is lacking in conventional homing experiments.

One solution lies in the following of the bird by light aeroplane or helicopter, and some work in this direction has already been reported by Griffin & Hock (1949). This method does not permit of following the bird for more than a few hours so that only the first one or two hundred miles of the course can be mapped, and the tracking of a bird which spends several days over the journey is obviously impossible. The method can only be applied to fairly large and conspicuous birds, is always open to the suspicion that the bird's flight is influenced by the proximity of the aeroplane, and is slow in gathering data. It is, nevertheless, a most powerful technique, and has already yielded invaluable information.

The importance of the flying time as opposed to the time between release and return was early realized by Exner (1905) who, in his important work on the homing of domestic pigeons, employed an ingenious device for its measurement. It consisted of a tube which was fixed to the bird and which contained some camphor. When the bird was not flying the rate of evaporation of the camphor was small, but when it flew the forced passage of air through the tube considerably accelerated the evaporation and the loss in weight of the camphor was taken as a measure of the flying time. The device was calibrated by whirling it on the end of an arm. This calibration, unfortunately, was very dependent on the temperature; for example an error of only 3° C., as between 8 and 11° C., in the estimate of the temperature

during a flight would have lead to an error of a factor two in the estimated flying time.

Essentially what is required is some form of clock which only operates when the bird to which it is fixed is on the wing and whose time-keeping is unaffected by such variables as the bird's air-speed and the ambient temperature. The clock should be very light so as not to interfere with normal flight, and, because of the large numbers required and the high probability of total loss, should be easy and cheap to make. Such clocks or flight recorders may easily be constructed, the mechanism being the recording, on a piece of photographic emulsion, of the α-particles emitted from a radioactive source. The rate of such emission is entirely unaffected by external circumstances while the recording is completely quantitative, examination of the processed emulsion through a high-power microscope showing very clearly the tracks of individual α-particles, enabling the total number received to be directly counted. (A popular account of the photographic plate technique for the studying of nuclear radiations such as the α-particle has been given by Powell & Occhialini (1947).) It is then merely necessary to combine such a radioactive source and photographic emulsion (both of negligible weight) with some sort of shutter which is interposed between the two except when the bird is in flight: the penetration of the α-particle into solid matter is only a few tens of microns so this shutter need not be very substantial. One possibility which immediately presents itself is to fix the recorder on to the bird in such a way that it is differently oriented when the bird is in flight or on the ground, and use gravity to open and close the shutter. The device which has been developed is illustrated schematically in Fig. 1 *a*, *b*. The orientation when the bird is not in flight is shown in Fig. 1 *a*. *S* is the α-particle source and *E*

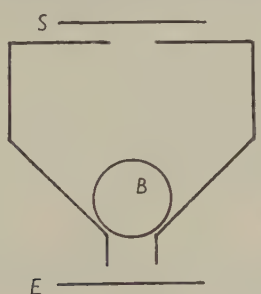

Fig. 1 *a*

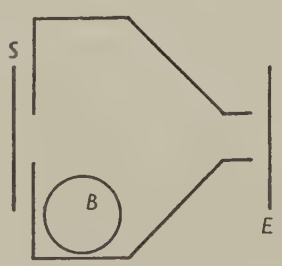

Fig. 1 *b*

the photographic emulsion; a steel ball *B* acts as the shutter and sits in the bottom of the 90° cone, preventing the α-particles from reaching the emulsion. *B* will remain in place until the recorder is tilted through more than 45° from this vertical position, so considerable latitude is allowable in the assumed orientation of the recorder at rest. When the bird is in flight the recorder takes up the orientation idealized in Fig. 1 *b*, *B* rolls out of the way and the α-particles record on the emulsion.

The legs would seem a good place to fix the recorder in order to achieve the effect of changing orientation, but not enough is known about their disposition under various conditions in all species to make this position of attachment reliable. The

wings are a natural second choice: it is easy to find places where the recorder may be fixed so that its orientation while the bird is on the ground is always within 45° of that of Fig. 1 *a* except in exceptional circumstances of violent motion, display and so on, while in flight the orientation of Fig. 1 *b* is approximated to. Thus in the domestic pigeon the recorder is fixed on the undersurface of the quill and inner web of the second primary just about where this feather disappears beneath the under wing coverts. At this point in normal carriage the quill is roughly horizontal, so the recorder is fixed at right angles to it. In flight the axis of the recorder is roughly horizontal and, in any case, the centrifugal force tends to hold the ball *B* against the side of the recorder rather than in the bottom of the cone.

The recorder is calibrated by laying it on its side as in Fig. 1 *b* for a known time. Tests with birds in the loft and in flight have shown that the device works accurately in both orientations. It has a fairly wide range of sensitivity. Thus in the model about to be described the area of emulsion exposed to the α-particle source is about 0·2 sq. mm.: within this area one can count quantitatively up to about 20,000 α-particle tracks (the tracks are formed at right angles to the plane of the emulsion so have the least tendency to overlap in the field of view). Now the counting of 1000 tracks gives a probable error, owing to statistical fluctuations, of about 2% in the estimate of the flying time, so a factor of 20 or so is permitted between the longest and shortest flight times if all are to be known to 2% or better, and if the accuracy aimed at were only 10% this factor would rise to 500. Any time scale from minutes to weeks may be catered for by changing the strength of the radioactive source, and even if 10,000 tracks are desired in a 10 min. flight the source strength demanded is less than a microcurie. The recorder may, of course, be stored all ready for use for long periods in the position of Fig. 1 *a* without any α-particle leakage on to the emulsion from the strongest source. The emulsion may be processed and examined at leisure.

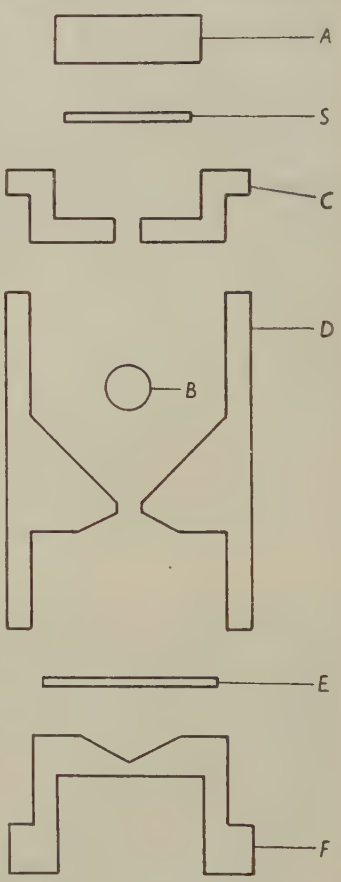

Fig. 2

An 'exploded' view of the working design of the recorder is shown in Fig. 2. *S*, *E* and *B* have the same significance as before. *S* is a polonium α-particle source deposited on a piece of thin silver foil from a solution in nitric acid. Polonium has the advantage of cheapness and of being a practically pure α-particle emitter—a source whose α-particles are accompanied by strong β or γ-radiation is undesirable because these radiations, more penetrating than the α-particles, may fog the emulsion if the device remains long before use and make counting of the α-particle tracks more difficult. The almost complete absence of γ-rays

means that there is no radiation hazard for the bird. A disadvantage of polonium is that it decays relatively quickly: unless all the flights and calibration exposures are over in a time much less than the half-life of 140 days the decay must be allowed for, but this may be done easily and accurately.* *E* is a disk of Ilford C 2 stripped emulsion of diameter 4 mm. and of thickness 50 μ. After exposure it may be affixed to a specially prepared glass slide supplied by Messrs Ilford, and then processed in the usual way. *B*, the steel ball, has a diameter of 1 mm., and serves as a scale for the figure. The rest of the device is made on the lathe, and is of duralumin. This metal is chosen for its lightness and because it does not have so deleterious an effect on the emulsion as many metals (anodized aluminium would be still better in this respect). The plug *F* is a push fit into the body *D*, and is short enough to allow a little play to the emulsion. *F* and *D* are recessed above and below the emulsion to avoid contact between the metal and the important central region of *E* where the α-particles strike. *C* may be cemented on to *D*; *A* may simply be coated on the sides with soft wax and pressed into *C* to retain the source *S*. The recorder, ready for use, weighs about 250 mg. It may either be fixed directly to the feather as described above, or, better, a light bucket made of duralumin may be fixed in its place some days before the flight and loaded with a mass equal to that of the recorder. The bird may then accustom itself to the attachment, and may be given practice flights with it in place. For use the recorder is slipped into the bucket and secured by a thread passing through a hole in the base of the bucket and in a slot filed along the diameter of *A* and *C*. A rubber solution, such as Bostik, has been found very satisfactory for sticking the device to the feather. Domestic pigeons accustom themselves within a few minutes to the attachment, and there is no evidence that their flight is affected in any way. A balance weight may be symmetrically fixed on the other wing. This device has been successfully used in the summer of 1949 in large-scale experiments on the homing of domestic pigeons performed in collaboration with Mr G. V. T. Matthews: these experiments form part of an extensive investigation into the homing and navigational ability of birds in progress at the Department of Zoology, Cambridge.

No attempt has been made to reduce the device to its smallest form. The size is

* The correction is made as follows. Suppose the bird spends a time τ_1 between release and return, the calibration exposure begins a time τ_2 after the release of the bird, and then it lasts a time τ_3; then the apparent flight time as deduced from the recorder should be multiplied by

$$\frac{\tau_1}{\tau_3}\frac{(1-e^{-\lambda\tau_3})}{(1-e^{-\lambda\tau_1})}e^{-\lambda\tau_2}.$$

If now $\lambda\tau_1$, $\lambda\tau_2$, $\lambda\tau_3 \ll 1$ this expression reduces to $1-\lambda(\frac{1}{2}\tau_3+\tau_2-\frac{1}{2}\tau_1)$, λ is the decay constant of the active body used: for polonium it is equal to 0·00495 day^{-1}, the τ's being measured in days.

This correction may, of course, be circumvented by using an active body of long half life (which is equal to $0{\cdot}693/\lambda$). Use of such a body would also get over the assumption, implicit in the correction stated above, that the flying time is roughly uniformly distributed throughout the time τ_1. Many such bodies of long half life are available, though none is so convenient as polonium.

It may be convenient, for some purposes, to use an α-particle emitter which decays with a half-life of only a few hours. This would then register the flying time over the first few hours after release only and speed of ultimate recovery would be unimportant. The carrying, by a bird, of two recorders, one containing a body of short and one of long half-life would then give a crude indication of whether the initial rate of search was maintained or altered in any way.

limited only by the availability of small steel balls or the equivalent, and by the inconvenience of handling small fragments of emulsion. An overall reduction in scale by a factor two should be realizable, and, with economy of metal, a mass of 20 mg. should be attainable. This would enable the technique to be applied to quite small birds.

It would be an advantage, for the study of gliding and soaring birds such as gulls and gannets, to have a device which distinguished between gliding or soaring and flapping flight. Such a composite device has not been made, but one which responds only to flapping flight is illustrated schematically in Fig. 3. A steel weight W of

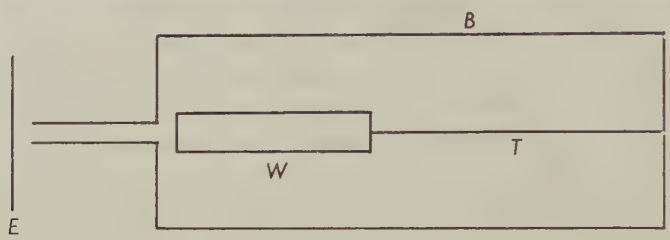

Fig. 3

length 4 mm., bearing on its free end an α-particle source is held axially within the cylindrical body B of the device by a thin tungsten wire T. When the device is not subject to sideways acceleration α-particles may pass down the narrow hole and register on the emulsion E. If accelerated, however, as when fixed to a flapping wing, the weight W moves from side to side relative to B, and the α-particles may only fall on the emulsion for a fraction of the time, while the free end of W is passing the hole. The recorder may be 'shut off' for storing by placing the pole of a magnet alongside, drawing W to the inner wall of B. A device of the proportions of Fig. 3 and with a wire of diameter 40 μ. has been made and tested on domestic pigeons. When fixed in the same place as described for the other device (but parallel to the quill), it recorded about one-fifth as many tracks when flying as when not. This fraction will vary with the species studied, and must be determined in test flights. If this can be done, an examination of the records of the two types of device carried by the same bird will reveal the time spent flapping, that spent gliding and that spent otherwise than in flight. This device has not yet been used in any large-scale experiments.

Refinements of the devices and applications of the method suggest themselves in numbers. Thus the combination of a compass card of emulsion and a shutter would yield the time spent flying in various directions. An air-speed indicator could be constructed by mounting the source on a light spring and vane. A device for measuring the time spent by a bird on water could be made by opposing a source to a piece of emulsion covered by a thin light and water-proof layer so that water came between the two when the bird was on, say, the sea. But such devices would demand much more technical skill in construction than those described here.

My thanks are due to Dr C. Waller, of Messrs Ilford Ltd., who supplied the disks of stripped emulsion upon which the work depended; to Dr A. G. Maddock, of the

Department of Radiochemistry, Cambridge, for his help with the polonium sources; to Mr D. D. Stewart, of the Cavendish Laboratory, for his patience and skill in machining the many prototypes of these devices, and, finally, to Mr G. V. T. Matthews, of the Zoological Laboratory, Cambridge, for much help with the tests.

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A QUANTITATIVE STUDY OF THE FRAGMENTATION AND HAEMOLYSIS OF MAMMALIAN RED CELLS BY HEAT

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(With Three Text-figures)

This paper is concerned with quantitative aspects of the fragmentation of red cells by heat and of the haemolysis which accompanies it. The former process involves a splitting of the cell into fragments which retain, at least in part, some of the properties of the intact cell (ability to swell in hypotonic media, ability to undergo certain shape transformations, etc.; see Ponder, 1949). The latter process results in the formation of ghosts with properties which vary with the haemolytic system; in some systems, the cell becomes a ghost by an all-or-none process (as in hypotonic systems containing cells in low volume centration, see Parpart, 1931), while in other systems the ghosts are partially haemoglobinized and partially rigid. To describe the phenomena adequately, it is accordingly necessary to consider the transition of the red cell to the ghost as a process which can occur in several ways in addition to the all-or-none escape of Hb and the replacement of the cell by an unsubstantial ghost; it is all the more important to do this because evidence is accumulating that red cell ghosts in several kinds of haemolytic system possess both shape and rigidity (Ponder, 1942, 1950, Lindemann, 1949*a*,*b*).

METHOD

The water-bath used for heating the cells consists of a highly insulated glass tank fitted with an immersion heater, an adjustable thermoregulator, and a stirring motor; another motor moves a rack-like tube holder to and fro in the water with an intermittent, jerky, motion at the rate of about 100 excursions of 1 cm. each per minute. Small glass tubes (75 × 10 mm.) contain 0·5 ml. of the blood or suspension to be heated; when the tubes are in place in the tube holder, the blood or suspension is immersed about 6 cm. below the surface of the water. The temperature of the bath can be kept constant to 0·05° C. by adjusting the thermoregulator.*

When the temperature of the bath has reached a selected temperature, e.g. 56° C., four to six small tubes containing the blood or suspension in which fragmentation is to be studied at that temperature are placed in the tube holder, and the shaking motor is started. After various times (3, 5 min., etc.) one of the tubes is removed and placed in an ice-bath for a few moments; cell counts and other determinations are then made on the contents of the tubes with as little delay as possible.

* The shaking mechanism was made for me by Mr Paul Cutajar of New York University Machine Shop. A convenient form of thermoregulator is one of a bimetallic type which can be quickly adjusted to any temperature over a wide range, and which operates without a relay.

The bath is now allowed to cool to some second desired temperature, say 54° C.; the thermoregulator is set for this new temperature, and four to six tubes containing the blood or suspension are again placed in the tube holder and are heated with shaking for selected times before removal to the ice-bath. The process is repeated at lower temperatures (52, 50° C., etc.), and in this way one obtains a series of systems in which the red cells have been exposed, with constant shaking, to various temperatures for various times.

A small sample of each system is drawn up in a red cell counting pipette, and the cells plus fragments on both sides of a haemacytometer chamber (Spencer Brightline) are counted. When fragmentation is extensive, the sample is diluted appropriately so that about 800 cells plus fragments are counted; the coefficient of variation associated with the imperfect distribution of this number is $\pm 3{\cdot}5$ %. So as to allow time for the smaller fragments to settle, the preparation is allowed to stand for about 5 min. in the counting chamber before counting is started. In order to avoid including ghosts in the count, the illumination is kept high, but, as will be seen below, there is often a question as to whether an object is a ghost or not, since haemolysis in heated systems is often not all-or-none. In some cases it is possible to count cells and fragments on the one hand and ghosts and incompletely haemoglobinized cells on the other; considerable judgement enters into making the counts, and the precision with which the same result can be obtained repeatedly on the same material increases with experience.

A sample of each system is transferred to a haematocrit tube (100 mm. with 1 mm. bore, sealed at the lower end), and is spun at 10,000 r.p.m. for 30 min. The volume concentration of the cells, fragments, and ghosts is measured in the usual way; the column of clear fluid overlying the packed mass is then removed, and the concentration of Hb in it is found photometrically and is expressed as a fraction of the concentration of Hb in the completely haemolysed system. If the former concentration, as a fraction of the latter, is C, the *quantity* of Hb present in the column of clear fluid above the packed cells, fragments, and ghosts is $p = C(1 - \rho_1)$, where ρ_1 is the volume concentration of the cells, fragments, and ghosts.

The upper part of the haematocrit tube, from which the clear Hb-stained fluid has been removed, is rinsed with saline introduced with a capillary pipette; this removes excess Hb from the walls of the tube. The haematocrit tube is then turned upside down and a one-hole rubber stopper is fitted over its closed end; supported in this stopper, it is suspended in a 100 × 13 mm. test-tube containing 1 ml. of water, and is spun slowly in the centrifuge. The spinning drives the cells, fragments, and ghosts from the inverted haematocrit tube into the water, where they haemolyse. The concentration of Hb in the solution which results is found photometrically, and is expressed as a fraction of the Hb concentration found by treating an unheated system similarly. If this concentration is C', the mean corpuscular Hb concentration of the objects in the packed column is $C'\rho_0/\rho_1$, where the volume concentration of the cells in the unheated system is ρ_0.

(1) *Fragmentation and the accompanying haemolysis*

The principal difficulty in the study of the kinetics of fragmentation is that haemolysis usually accompanies it, and that the haemolysis, unlike that in many haemolytic systems, is not necessarily all-or-none. This introduces severe limitations on the extent to which the phenomena can be dealt with quantitatively, and it will be convenient at the outset to see what these limitations are.

When a red cell, or the fragments into which a red cell breaks, loses a fraction a of its initial Hb, its volume may or may not decrease in proportion. Let the change in volume with change of a be $dv/da = k$; then $v = 1 - ka$ if the initial volume is denoted by unity. The amount of Hb which the cell loses is a, and this appears in the supernatant fluid of the system; the fraction $(1 - a)$ is left in the cell, and the corpuscular Hb concentration is always $(1 - a)/(1 - ka)$. In the case of individual cells, there are at least four possibilities to be considered.

Case 1. Complete loss of Hb, complete loss of volume; all-or-none haemolysis. Here $a = 1$, $k = 1$.

Case 2. Complete loss of Hb, no loss of volume; rigid ghosts. Here $a = 1$, $k = 0$. The corpuscular Hb finally becomes the same in concentration as that in the fluid surrounding the cells.

Case 3. Partial loss of Hb, no loss of volume; partial haemolysis or retention of Hb depending on whether the process is looked at from the standpoint of the cell or from that of the ghost. Here $a < 1$, $k = 0$. The corpuscular Hb concentration never becomes as low as that in the surrounding fluid.

Case 4. Partial loss of Hb, accompanied by loss of volume; small haemoglobinized ghosts. Here $a < 1$, and k can have values between 1 and 0. When $k = 1$, the corpuscular Hb concentration remains unity throughout the process; when $k < 1 > 0$, the corpuscular Hb concentration is less than unity, but never becomes as small as that in the surrounding medium.

When observations are made on populations of red cells before and after heating, the results cannot always be reduced to one or another of these simple cases. In such systems we observe p, the fraction of the total Hb liberated into the supernatant fluid obtained by packing cells, fragments, and ghosts, and also ρ, the volume concentration of these packed objects. In place of the corpuscular Hb concentration, we now have the mean corpuscular Hb concentration of the cells, fragments, and ghosts; this is $C_m = (1 - p)/(1 - ka)$, and the change in volume with loss of Hb is now $d\rho/dp = k$. Using these values, an attempt can be made to reduce the observations to one or another of four cases which correspond to those enumerated above.

Case 1*a*. Complete loss of Hb, complete loss of volume; all-or-none haemolysis. Plotting ρ against p gives a straight line with a slope of $-\rho_0$, which makes intercepts $\rho = \rho_0$ and $p = 1$. Determinations of C_m show that it has the value of unity for all values of p. This case is illustrated by the behaviour of human red cells when heated to increasing temperatures or for increasing times (Fig. 1, 1*a*). Haemolysis as measured by p increases and ρ decreases linearly with it, complete haemolysis corresponding to a negligible volume of ghosts.

Case 2*a*. Complete loss of Hb, no loss of volume; rigid ghosts. Plotting ρ against p gives a straight line parallel to the p-axis, i.e. with a slope of zero, and terminating in a point the co-ordinates of which are $\rho = o_0$, $p = 1 - \rho_0$. This is the largest value of p which can occur in the system, because it is the value which makes the concentrations of Hb inside and outside the ghosts equal. When determined at this value of p, $C_m = \rho$, and this is the lowest value it can reach. Geometrically, the co-ordinates of the terminal point form a square with the p-axis and the vertical at $p = 1$ (Fig. 1, 2*a*). There is no example of this process occurring in heated systems as affecting all of the cells, but it is met with as a part of case 5, below.

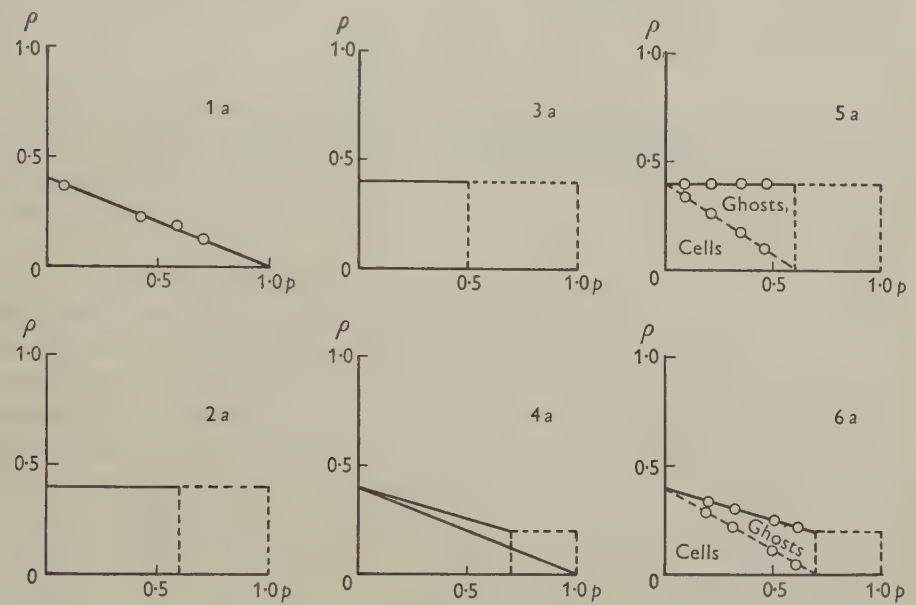

Fig. 1. To illustrate various possible relations between ρ and p; for description, see text. Experimental results are shown as circles. 1*a*, washed human red cells; 5*a*, washed sheep red cells; 6*a*, washed human red cells, lecithinated. Other cases (2*a*, 3*a*, 4*a*) not met with experimentally in this investigation.

Case 3*a*. Partial loss of Hb, no loss of volume; partial haemolysis or retention of Hb. Plotting ρ against p gives a straight line parallel to the p-axis, but terminating in a point for which p is less than $1 - \rho_0$. A rectangle, and not a square, is formed by the co-ordinates of the point, the p-axis, and the vertical at $p = 1$. The concentration of Hb inside the ghost never falls as low as that in the surrounding medium, and C_m is always greater than ρ_0 (Fig. 1, 3*a*). There is no example of this process occurring in heated systems as affecting all of the cells, but, like case 2*a*, it enters into the description of compound cases, below.

Case 4*a*. Partial loss of Hb, accompanied by loss of volume; small haemoglobinized ghosts. Plotting ρ against p gives a straight line with a slope between zero and $-\rho$, ending in a terminal point with co-ordinates ρ_1 and $1 - \rho_1$, where ρ_1

is the (diminished) volume of the ghosts. C_m determined at this value of p exceeds ρ_1 but is less than unity, so that the concentration of Hb in the ghost never falls to that in the surrounding medium, and may be considerably above it (Fig. 1, 4*a*). Like cases 2*a* and 3*a*, there is no example of this process as affecting all the cells of a heated system.

In experiments with populations of cells, complex cases are met with over and above those which arise in the case of single cells. If there are N cells in the system and if each can lose a fraction a of its Hb, the Hb lost is $p=Na$, but the same value of p can result from some of the cells having lost all of their Hb or from all of the cells having lost some of their Hb. The situation in the partially haemolysed system can thus be represented, generally, by

$$p=N_1a_1+N_2a_2+N_3a_3, \qquad (1)$$

where N_1, N_2, N_3, etc., are fractions of the total number N which behave in different ways as regards the loss of their pigment, as indicated by the different values of a. Each fraction may also behave differently as regards the loss of its volume, and so a large number of possible situations arise. Two of these are of particular interest in connexion with heated systems.

Case 5*a*. There are only two classes, N_1, the cells which haemolyse, and $N_2=(N-N_1)$, those which remain intact. For the latter, $a_2=0$, and for the former $a_1=1$, $k=0$. The value of p increases because the value of N_1 increases, more and more cells losing all of their Hb to give the increase in p. This case is to be contrasted with case 2*a*, in which all the cells lose more and more of their Hb as p increases. To distinguish between them we have to fall back on other criteria; when all the cells lose part of their Hb, the column of packed objects becomes uniformly paler as p increases, but when some of the cells lose all their Hb, the remainder losing none, the column of packed objects is likely to show a contrast between haemoglobinized intact cells and pale ghosts, a decrease in the volume concentration of the cells being accompanied by an increase in the volume concentration of the ghosts. This is illustrated by the behaviour of rabbit or sheep red cells on heating, and shown in Fig. 1, 5*a*. Further, in case 2*a*, examination under the microscope shows more or less uniform objects which become paler as p increases, whereas in case 5*a*, the field shows two categories of object, a fully haemoglobinized cell and a pale but voluminous ghost.

Case 6*a*. Again there are only two classes, N_1, the cells which haemolyse, and $(N-N_1)$, those which remain intact, but for the former a_1 and k_1 have values between 1 and 0, i.e. some cells haemolyse in a partial manner in Barón's (1928) sense of the term. In this case the value of p increases both because N_1 increases and because a_1 increases, and is always greater than can be accounted for by the diminution in ρ, so that plotting ρ against p gives a line such as that shown in Fig. 1, 6*a* (experimental data derived from heated human red cells treated with lecithin). Examination of the preparation with the microscope shows intact cells, decreasing in number as p increases, together with ghosts and fragments with varying degrees of haemoglobinization.

Finally, the best measure of the average number of fragments which each fragmenting red cell breaks into is

$$f = \frac{N_f/N}{1 - N_{a=1,\,k=1}}, \tag{2}$$

where N is the initial number of cells per unit volume as seen on the counting chamber, N_f the number of cells, fragments, and visible ghosts (possessing volume and also haemoglobin, whether by reason of its being 'surplus Hb' or merely 'equilibrium Hb' trapped in a voluminous ghost which has undergone some shrinking), and where $N_{a=1,\,k=1}$ is the fraction of N which undergoes all-or-none haemolysis, e.g. which leaves no ghosts. If haemolysis is all-or-none, $N_{a=1,\,k=1}$ is measured by p. If haemolysis is of the type described by cases $2a$, $3a$, $4a$, $5a$ or $6a$, $N_{a=1,\,k=1}$ is zero, and f is simply N_f/N. In experiments with populations of red cells, situations may arise in which it is very difficult or impossible to determine what value ought to be given to $N_{a=1,\,k=1}$, but no expression more simple than expression (2) seems to be a consistent measure of the amount of fragmentation. Fortunately, most systems in which fragmentation is produced by heat are examples either of case $1a$ or of case $5a$.*

(2) *Fragmentation as a function of time and temperature*

Fragmentation as measured by f in expression (2) increases with the time during which the cells are kept at any one temperature, and increases as the temperature is increased. Fig. 2 shows a series of curves for human red cells, f being plotted against t at three different temperatures. The precision of the points, which is none too great under any circumstances because of the errors associated with the counting, is still further reduced by the heating of the cells not being instantaneous; it requires a short time for the systems in the small tubes to reach the temperature of the water-bath, and so the real value of t is always smaller than the recorded value. An attempt has been made to compensate for this by subtracting from all values of t the time required for the cells to reach 48° C., the temperature in the neighbourhood of which fragmentation begins; these small corrections affect the shorter times more than they do the longer ones.

At any one temperature, the relation between t and f seems to be a curve concave to the t-axis and proceeding towards an asymptote at $t = \infty$. A situation in which some of the cells fragment before others would tend to give rise to a sigmoid relation between t and f, but there are several secondary effects which obscure such a relation, e.g. a tendency for the more easily fragmented cells to break into a larger number of fragments than are derived from the cells which fragment with more difficulty. Perhaps for this reason, no evidence of a sigmoid relation has been obtained. As the situation stands, there is nothing to be gained by attempting to find an expression

* Increases in the volume of intact red cells as a result of heating have not been considered in the foregoing discussion. In some systems these are not only noticeable, but troublesome, systems containing heated cells giving greater values of ρ than otherwise identical systems containing unheated cells, even when some lysis occurs in the heated systems. This effect can be minimized by using as ρ_0 the volume concentration for a system which has been heated to a temperature a few degrees below $T_{0,F}$, and then plotting ρ against p, as above.

which will fit the curves, since by analogy with the haemolytic process, this expression would almost certainly have to take account of a variation in resistance to fragmentation, of the number of fragments into which a cell of given resistance breaks, and also of the velocity of whatever 'fundamental reaction' underlies the fragmentation.

The values of f and of p corresponding to the asymptotes, on the other hand, are related to the temperature T in a simple way. Call the particular values of f and p which correspond to the asymptotes F and P; plotting them against the temperature T gives two good straight lines (Fig. 2, inset) with slopes K_F and K_P, making

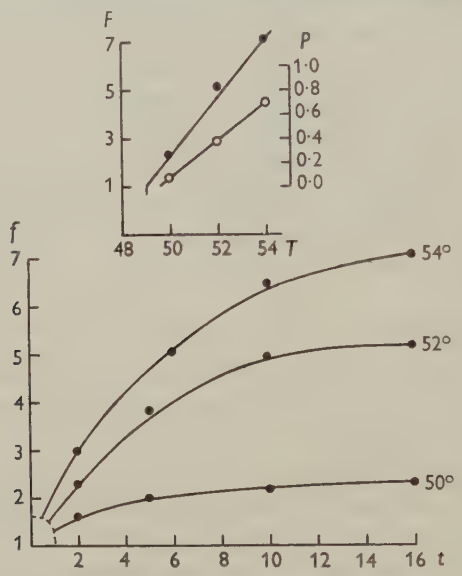

Fig. 2. The relation of the extent of fragmentation, f, to time t in minutes at three different temperatures. Washed human red cells. Inset: F (dots), the value of f at the asymptotes, and P (circles), the value of p at the asymptotes, plotted against temperature T. The intercepts on the line $F=1$ and $P=0$ are $T_{0,F}$ and $T_{0,P}$ respectively.

intercepts $T_{0,F}$ and $T_{0,P}$ respectively on the line $F=1$, $P=0$. The equations accordingly are

$$F=K_F(T-T_{0,F}), \tag{3}$$

and

$$P=K_P(T-T_{0,P}). \tag{4}$$

By means of these expressions, the fragmentation and haemolysis which occur at the end of long times of heating (15 min. being considered as indefinitely long in these experiments) can be expressed in terms of four constants, two for each process. The temperature $T_{0,F}$ is the highest temperature to which the system can be heated without fragmentation occurring, and the temperature $T_{0,P}$ is the highest temperature to which the system can be heated without haemolysis occurring; both values are obtained by extrapolation. In the case of washed human red cells, $K_F=1{\cdot}22$, $T_{0,F}=49°$ C., $K_P=0{\cdot}15$, and $T_{0,P}=49{\cdot}6°$ C. Extrapolation also gives a temperature at which $P=1{\cdot}0$, i.e. at which complete haemolysis occurs after 15 min. heating; in the case of washed human red cells, this temperature is about 56° C. It would

probably be a mistake, however, to attach much significance to this value or to the corresponding value of F (=9·6), because it is doubtful whether the relation between F and T and between P and T remains linear when the value of P is greater than about 0·7.

(3) *Fragmentation of human red cells by heat; experimental modifications of the process*

The types of fragmentation which occur in preparations of human red cells in plasma have already been described as being of three kinds (Ponder, 1949): the appearance of radial defects from a central thinned-out area, with the subsequent breaking of the cell into fragments bounded by these defects, the development of irregularities at the cell margin and the fragmentation of the cell as a result of the extension of defects originating from the bases of these irregularities, and a 'beading' of the cell rim with the development of fragments from the 'beads'. The fragmentation of washed human red cells occurs in essentially similar ways, except when the cells are rendered spherical by being placed between slide and cover-glass; under these conditions they become spherical and haemolyse without fragmenting as the temperature is raised.

The three principal conclusions of this investigation can be illustrated by the comparison, in a quantitative way, of heated systems containing (1) fresh human red cells, freshly washed, (2) human red cells which have been kept at 4°C. for several days and then washed, (3) human red cells, washed and then treated with distearyl lecithin, and (4) fresh human red cells in plasma. The results illustrate the conclusions that heating under different conditions may give rise to ghosts with different properties (comparison of 1 with 3), that conditions which tend to render the cells spherical prevent its fragmentation (comparison of 1 with 2 and 3), and that plasma contains substances which inhibit both fragmentation and heat haemolysis (comparison of 1 with 4).

(i) *Fresh human red cells, freshly washed.* In this system, haemolysis is all-or-none (Fig. 1, 1*a*), and so F can be found from expression (2) with $N_{a=1,\ k=1}=P$. The straight line which results is shown in Fig. 3 (man, case 1*a*), together with the experimental relations from which it is derived, these being a linear relation between P and T and a relation between N_F/N and T, the latter passing through a maximum. In this system, fragmentation begins at a lower temperature than that at which haemolysis begins, and at a temperature such as 53° C. the number of objects seen on the counting chamber is about three times the initial number; all of these, moreover, are cells or haemoglobinized fragments ($C_m=1$ for all values of P), the ghosts being invisible under the conditions of illumination used.

(ii) *Human red cells, kept at* 4° C. *for* 48 *hr.; then washed.* Again haemolysis is all-or-none, and F is found as in the preceding case. The keeping of the cells for 48 hr. at 4° C., however, enhances the haemolytic aspect of the heating effect, so that the relation between P and T is represented by very nearly the same straight line (although on a different scale) as that which represents the relation between F and T (Fig. 3, man, 48 hr. at 4° C., case 1*a*). At the same time, the relation of

N_F/N passes more sharply through its maximum, and at a temperature such as 53° C. the number of objects on the counting chamber is no greater than the initial number. This is due to the preliminary storage having increased the haemolysis of fragments at any given temperature. That this is probably the result of an effect on red cell shape is suggested by what is seen when the heating chamber (Ponder, 1949) is used to compare a system containing fresh cells with a system containing stored cells. In the latter, instead of defects originating centrally or peripherally and extending to split the cell into fragments of approximately equal size, small irregularities at the edge of the cell round up to form a number of minute fragments surrounding the remainder of the cell, which at the same time becomes spherical. As the temperature is increased, this sphere haemolyses without further fragmentation. The effect of storage is thus essentially an effect on red cell shape, a process involving sphere formation and haemolysis taking the place, to some extent, of the fragmentation process seen in the case of the washed discoidal red cells of fresh blood.

(iii) *Human red cells, washed and treated with lecithin.* These systems are prepared by washing human red cells with saline and then adding a sufficient quantity of a sol of distearyl lecithin (1 mg./ml. in saline) to bring the volume concentration to 0·4. After standing for 15–30 min. at room temperature, the cells appear between plastic surfaces as spheres or finely crenated spheres.

When heated, systems treated with distearyl lecithin show partial haemolysis (case 6*a*), the ghosts being haemoglobinized to different extents. The volume of the ghost is less than that of the intact cell, but all the ghosts remaining are clearly visible objects on the counting chamber; the value of F is accordingly calculated as N_F/N. As the temperature is increased, N_F/N and F remain virtually constant, i.e. there is no fragmentation, but P increases (Fig. 3, man, lecithin, case 6*a*). This is a very clear case of haemolysis taking the place of fragmentation when the shape of the cell is changed from that of a disk to that of a sphere.

(iv) *Fresh human red cells in plasma.* The effect of heat on the cells of these systems differs greatly from that observed in systems containing washed red cells (Fig. 3, lower figure). Both fragmentation and haemolysis are less conspicuous when plasma is present, as may be seen by comparing the curves marked F, saline with the curve marked F, plasma, and the curve marked P, saline with the curve marked P, plasma. The relation between fragmentation and temperature is not linear in systems containing plasma, at least when F is calculated on the basis of the haemolysis being all-or-none. The situation will probably require much further investigation before it is clarified, but the presence or absence of plasma apparently has about the same effect on both fragmentation and haemolysis as a 4–5° C. difference in temperature (the value of F at 51° C. in the absence of plasma is roughly equal to the value of F at 56° C. in the presence of plasma, and the value of P at 52° C. in the absence of plasma is roughly the same as the value of P at 56° . in the presence of plasma). It can also be shown that the remarkable inhibitory or protective effect of plasma on fragmentation haemolysis is largely due to the contained serum albumin, and it will be recalled that serum albumin is an anti-sphering substance responsible for the maintenance of the discoidal form of the red cell in many systems.

These results may be clearer if considered in terms of a simple hypothesis. Fragmentation by heat is apparently an event which takes place during a transition of the red cell from a body with some degree of elasticity to a plastic body, and finally to a body which behaves as if it were a highly viscous fluid. If the cell is spherical, there is virtually no tendency to fragmentation, for the sphere itself is a very stable form, and the more nearly the cell approaches to a sphere, the more stable it is. If the cell is a disk, on the other hand, it may become unstable at some stage of the transition from its initial state to the final state of a viscous fluid. In the case of a cylinder of highly viscous material, instability usually occurs when the length of the cylinder is $2\pi r$, r being the radius of the cylinder. Ignoring the material which fills the biconcavity and which becomes attenuated when the cell is about to fragment, the discoidal red cells can be regarded as the body generated by a circle of radius r moving in a circle of radius R, $(R+r)$ being the semi-diameter of the cell and r being half the thickness of the rim. Since $2\pi R > 2\pi r$, a viscous body of this shape will become unstable for the same reasons as a cylinder of the same material becomes unstable, and will tend to break into beads or fragments. The temperature $T_{0,F}$ at which this occurs, however, will be that at which the properties of the materials of which the cell is composed change from those of an elastic or plastic solid to those of a highly viscous fluid, and so $T_{0,F}$ will be determined by the particular architecture of the red cell ultrastructure. It is easy to understand that the presence of plasma can change $T_{0,F}$ substantially, for the presence or absence of plasma components has a very substantial effect on the surface ultrastructure at least, as shown by their effect on red cell shape.

(4) *Fragmentation of the red cells of other mammals by heat*

The conclusions that heating may result in the production of ghosts with differing properties, and that conditions which tend to render the cells spherical prevent its fragmentation, can be further illustrated by examining the fragmentation and haemolysis of the red cells of mammals other than man. Speaking somewhat generally, the value of K_F is determined by the shape of the cells, for K_F is zero when the cells are spherical and larger when the cells are flat disks *at the temperature at which fragmentation takes place;* the value of $T_{0,F}$, on the other hand, is determined by properties which have no necessary relation to shape, but which determine when the cell begins to behave as a viscous fluid instead of as an elastic or plastic solid. The properties of the ghosts, e.g. whether they possess rigidity or not, are again likely to be related to the architecture of the ultrastructure of the type of cell under consideration; the study of heat fragmentation of the red cells of different mammals may accordingly be expected to reveal a situation which is partly simple and partly complex.

Table 1 shows representative values for K_F and for $T_{0.F}$ found for the red cells of man, rabbit, dog, cat, ox and sheep, this order being that of decreasing red cell diameter and volume. The relatively flat red cells of man, the rabbit, the dog, and the cat remain flat up to temperatures at which fragmentation occurs and give values of K_F which range from 1·22 to 0·25; the values do not altogether correspond to the

red cell diameters or volumes, but K_F is a constant which is easily affected by minor variations in the state of the cells, e.g. on the length of time after withdrawal from the animal (cf. Fig. 3), and so the correspondence is probably as good as one would expect. The same tendency for these relatively large and flat cells to undergo

Table 1

Animal	K_F	$T_{0,F}$
Man	1·22	49·0
Rabbit	0·50	52·0
Dog	0·25	53·0
Cat	0·34	51·5
Ox	0·01	60·0
Sheep	0·02	55·5

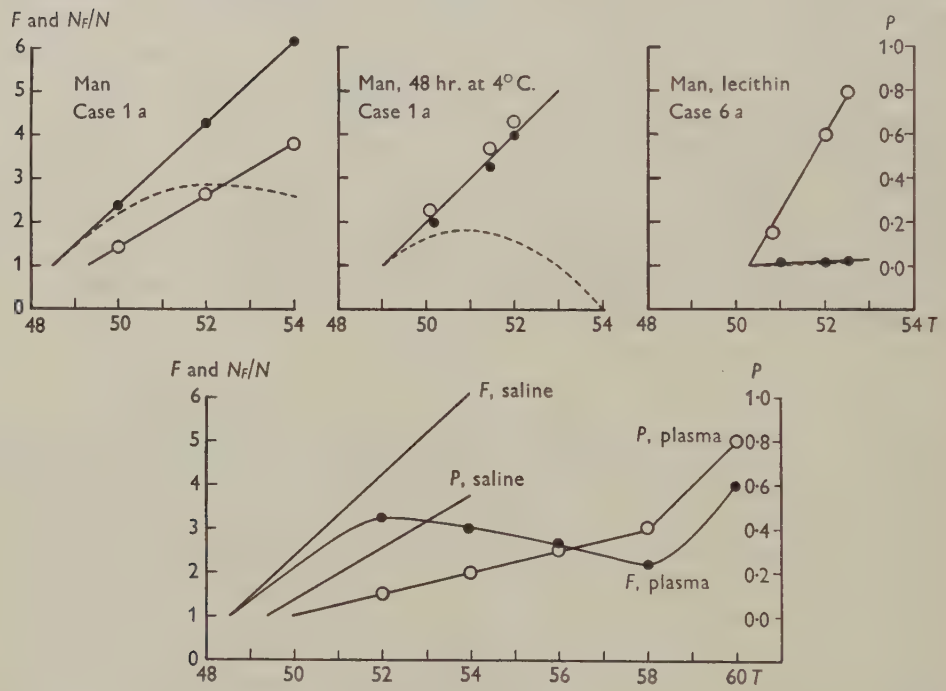

Fig. 3. The variation of F (dots), P (circles), and N_F/N (dotted line) with temperature T (abscissa). Upper left, fresh human red cells, freshly washed; upper middle, human red cells, kept at 4° C. for 48 hr., then washed; upper right, human red cells, washed, and treated with lecithin. Lower, fresh human red cells in saline and in plasma. For description, see text.

fragmentation is seen when they are observed directly on the heating chamber; one is struck by the fact that any crenations which are present initially tend to disappear as temperatures in the neighbourhood of 45° C. are reached, and that the cells of the rabbit, dog, and cat undergo fragmentation at higher temperatures in much the same way as human red cells do, although the number and size of fragment per cell is smaller. The red cells of the ox and sheep, on the other hand, are less flat to begin

with; they become spherical at temperatures in the neighbourhood of 50° C., and at higher temperatures they haemolyse almost without fragmentation. It is an interesting and unforeseen result that $T_{0,F}$ increases as K_F decreases; this presumably means that both constants measure related attributes of the architecture of the red cell ultrastructure.

Heat haemolysis of the red cells of man and of the dog is substantially all-or-none; haemolysis of the red cells of the rabbit, the cat, and the sheep, on the other hand, is an example of a process which is described either by case 5*a* or 6*a*, i.e. the ghosts have rigidity, complete or partial, and are partially haemolysed. The reason for these differences in the properties of the ghost remains to be investigated.

SUMMARY

A method is described by means of which the fragmentation and haemolysis resulting from heat can be studied quantitatively, and by means of which certain situations as regards the rigidity and the extent of haemoglobinization of the ghost can be distinguished from each other.

Fragmentation and haemolysis increase with time so as to become virtually complete after 15 min. heating at any temperature. The fragmentation and haemolysis at the end of 15 min. is linear with the temperature, and so can be expressed in terms of four constants, two referring to the former process and two to the latter.

Heating of human red cells under different conditions may give rise to ghosts with different properties as regards rigidity and degree of haemoglobinization. Conditions which render the cells spherical prevent its fragmentation, and plasma contains substances (albumins) which inhibit both fragmentation and haemolysis by heat. Similar conclusions can be reached by examining the heat fragmentation and haemolysis of the red cells of mammals other than man.

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NITROGENOUS EXCRETION OF AMPHIPODS AND ISOPODS

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INTRODUCTION

It is generally agreed that the most primitive type of nitrogenous excretory product, arising directly from the α-amino-N of proteins by deamination, is ammonia. In an extensive survey of the invertebrates, Delaunay (1927) was able to account for more than 50% of the total non-protein nitrogen (N.P.N.) excreted in terms of ammonia in a large number of unrelated aquatic species, including the sipunculid, leech, crayfish, crab and cuttlefish. Delaunay showed further that the nitrogenous excretion of terrestrial invertebrates is closely analogous to that of terrestrial vertebrates, in that the predominant end-product may be either urea or uric acid. He suggested that the synthesis of these more complex molecules should be regarded as an adaptive mechanism for the detoxication of ammonia, necessitated by a reduced circulation of water through the body: 'Les processus d'uréogénèse et d'uricogénèse... représentent des mécanismes chimiques d'adaptation qui évitent l'accumulation de corps ammoniacaux toxiques dans le milieu intérieur et sont ainsi nécessaires pour la vie terrestre' (Delaunay, 1934). This concept of a correlation between environmental conditions and the nature of the main end-product of nitrogen metabolism has found much support, as is shown, for example, in a recent comprehensive review by Florkin (1945).

Adaptations to terrestrial conditions have been acquired independently in several phyla, including the annelids, molluscs, arthropods and chordates, and in all cases so far studied ammonia is replaced at least in part by some less toxic end-product, usually urea, uric acid or even guanine. In order to obtain a closer insight into the correlation of the nitrogenous excretion with habitat, it is particularly relevant to study those groups which morphologically are closely related and yet distributed over a variety of habitats. Interesting results have been obtained in this connexion for the chelonian reptiles and gastropods. In the case of the Chelonia, Moyle (1949) was able to demonstrate a biochemical separation into three groups closely corresponding to three types of environment in which these reptiles are found. In the excreta of semi-terrestrial species urea predominates, whereas the desert forms are essentially uricotelic, although the excretion of urea has not been entirely suppressed. The secondarily aquatic forms, on the other hand, have to some extent reverted towards ammonotelism, urea and ammonia being produced in approximately equal quantities.

The problem of nitrogen katabolism in gastropods (Needham, 1935) was investigated indirectly by a study of the uric acid content of the nephridia and other tissues on the supposition that a uricotelic metabolism would be associated with some retention of this end-product. It was found that the uric acid content is high in terrestrial as opposed to aquatic forms, and although significant amounts of uric acid were present in a few aquatic species, in most of these a terrestrial ancestry can be traced on morphological grounds.

The class Crustacea is primarily aquatic, but certain representatives of the Isopoda and Amphipoda inhabit different levels of the sea-shore while others, the woodlice, have established themselves on land. Since the nitrogenous excretion of these animals appears to have received no attention so far, if was felt that a detailed study of this small and comparatively uniform group was particularly desirable. A survey of the nitrogenous excretion of a variety of species of amphipods and isopods, including marine, fresh-water and terrestrial forms, was therefore undertaken.

Table 1. *Description of amphipod and isopod species investigated*

Species	Order	Habitat	Locality	Experimental medium
Gammarus locusta (1)	A	ML	Near low-water mark among weeds and under stones	100% SW
Marinogammarus marinus (2)	A	ML and E	In association with *Gammarus locusta*, also higher up on the shore and in estuaries	80% SW
Marinogammarus pirloti (2)	A	ML	Near high-tide mark, sometimes in small streams where conditions alternate between the salinity of undiluted sea water and practically pure fresh water. Also found in large clusters under completely exposed stones	100% SW, 60% SW and 30% SW
Gammarus zaddachi (1)	A	E	In upper regions of estuaries; able to tolerate very low salinities	30% SW
Orchestia sp. (shore-hopper)	A	ST	At high-water mark, under weeds and stones	100% SW
Ligia oceanica (shore-slater)	I	ST	Just above high-water mark in rock crevices; sometimes in association with terrestrial woodlice	100% SW
Oniscus asellus (3)	I	T	The most primitive of the terrestrial isopods, restricted to damp environments; most frequently beneath bark of rotting wood	—
Porcellio laevis (3)	I	T	Dampish environments, usually among vegetable rubbish near domestic dwellings	—
Armadillidium vulgare (3) (common pill woodlouse)	I	T	Under wood and stones; able to withstand drier habitats than any other of the species studied, possibly due to its ability of rolling up in a ball	—
Gammarus pulex	A	FW	In slow-moving streams	Tap water
Asellus aquaticus	I	FW	In mud and weed of slow-moving streams and stagnant ponds	Tap water

For species nomenclature see (1) Spooner (1947), (2) Sexton & Spooner (1940), (3) Webb & Sillem (1906). Habitats are represented by ML, marine littoral; E, estuarine; ST, semi-terrestrial; T, terrestrial; FW, fresh water. SW denotes sea water; in the case of the semi-terrestrial species this was used for moistening the dish only. A, I, denote amphipod and isopod respectively.

MATERIAL

Since large numbers of animals were required in order to obtain sufficient amounts of nitrogenous excreta for analysis, it was possible to study only the most commonly occurring species. The marine littoral and estuarine forms were collected in the neighbourhood of Plymouth, while the terrestrial and fresh-water species were obtained in Cambridgeshire. A description of the species studied is given in Table 1.

ANALYSIS OF EXCRETA

Collection of excreta

Freshly collected animals were used whenever possible, in order to avoid the problems of feeding and keeping the animals in a healthy condition in the laboratory. The excreta were usually collected for one or two consecutive periods of 24 hr., during which time the animals received no food.

The aquatic species were kept in a cool, dark place in enamel dishes covered with lids and containing shallow water of the composition indicated in Table 1. Where sea water was used, this was previously passed through a bacterial filter-candle; micro-Kjeldahl determinations showed that it was then entirely free from nitrogenous substances. At the end of each experiment the water was filtered to remove the faeces, moulted skins and other debris. In several cases the solid matter thus removed was extracted and the extracts tested for uric acid, the most insoluble nitrogenous end-product likely to be encountered. Consistently negative results were, however, obtained, and it seemed improbable, therefore, that any nitrogenous excretory matter was removed by this preliminary filtration. The filtrate, usually 200–250 ml., was acidified to about pH 4 (acid to bromcresol green) and then concentrated on a boiling water-bath to a final volume of 20–50 ml. All the substances estimated were found to be stable to this treatment with the exception of uric acid, which is destroyed completely. For estimations of this compound it was therefore necessary to concentrate a separate small portion of the original sample under reduced pressure. Preliminary experiments showed that uric acid can be totally recovered if a sample of 50 ml. is concentrated to 5 ml. provided the temperature of the water-bath does not exceed 60° C.

The semi-terrestrial forms proved rather more difficult to handle. Nicholls (1931) appears to have kept *Ligia* alive during several weeks of total submersion, but in our own experiments casualties were rather high even during 24 hr. This may have been due to the inevitable crowding. The animals were therefore kept in moistened enamel dishes covered with dark lids, and washed before and after the experiment. The washings of the dish and animals after the experiment were collected for analysis. Preliminary experiments, in which the animals were placed in a large conical flask with a circulation of air passing through, showed that no volatile ammonia is lost in this way. In the case of *Orchestia*, the shore-hopper, the difficulties of keeping the animals alive during the experiment proved even greater. Complete submersion invariably resulted in a high mortality, and it was difficult to prevent

the animals from escaping if kept in any other way. Even when they were placed in a moist breffet with a few clean stones the casualties often amounted to 20%. The most successful method, which, unfortunately, was adopted only in the last few experiments, was to line the bottom of the breffet with several layers of damp muslin and a few clean stones. The animals, stones and muslin were washed thoroughly at the end of the experiment.

The terrestrial species were kept in glass dishes inside closed containers through which air was circulated. Before entry into the dish the air was bubbled through dilute sulphuric acid to remove any volatile bases, and after exit through a solution of boric acid to trap any volatile ammonia produced during the experiment. It was found that some 10–30% of the total ammonia excreted was usually given off in a volatile form. Before the experiment the animals were washed and dried on filter paper. They were again washed after the experiment in order to collect any adhering excretory material. The solid faecal pellets were scraped out of the dish, ground up with very dilute hydrochloric acid in a mortar, mixed with the washings of the dish and animals, brought rapidly to the boil, cooled and filtered. A control experiment showed that no uric acid is lost in this way. The filtrate was concentrated if necessary.

All samples of excreta which were not analysed immediately were stored in the refrigerator after addition of a few drops of chloroform. However, the possibility of bacterial involvement during the course of the experiments is a factor to be reckoned with but difficult to allow for. Since the experiments were conducted at relatively low temperatures and for a period of not more than 1 day, we do not consider that bacterial interference can have appreciably altered the true nitrogen partition data.

Analytical methods

Protein precipitation was carried out by the method of Folin & Wu (1919). Care was taken to ensure that the final pH of the solution was more acid than pH 2·8 (Merrill, 1924).

Ammonia N was estimated by steam distillation in Markham's apparatus (1942). The distillate was collected in a 2% (w/v) boric acid solution containing the mixed indicator recommended by Conway & O'Malley (1942) and subsequently titrated with N/70-HCl. This method is not entirely specific for ammonia, since any other volatile bases present will be included in the estimation. The only other volatile base which might reasonably be expected in this material is trimethylamine, particularly as Norris & Benoit (1945) have demonstrated the apparently universal occurrence among members of the Crustacea of trimethylamine oxide. However, the peculiarly repugnant smell of trimethylamine was never observed, and we believe that the volatile N was, in fact, ammonia throughout and refer to it as such.

Total N was estimated by the micro-Kjeldahl method. Samples were incinerated for 4 hr. with 2 ml. conc. H_2SO_4 and the catalyst of Chibnall, Rees & Williams (1943), and the ammonia thus formed was estimated by distillation in Markham's apparatus (1942). When high concentrations of salt were present in the sample, care had to be taken to remove the HCl by vigorous boiling in the presence of conc. H_2SO_4, and rather more conc. H_2SO_4 was usually added in these cases. Even so, it was sometimes

found that a little HCl distilled over into the boric acid, and it was then necessary to redistill the distillate.

Urea N was estimated as ammonia by steam distillation in Markham's apparatus (1942) after incubation with the urease extract of Cole (1937) at about pH 5 (purple coloration with 3 drops bromcresol green and 4 drops methyl red indicator solutions). In the presence of high concentrations of salt the urease tended to become inactivated, and in such cases an equal volume of water was added to the sample prior to incubation.

Amino-acid N was determined by treatment with ninhydrin at pH 2·5 according to the method of Sobel, Hirschman & Besman (1945). The ammonia thus liberated was steam distilled as described above instead of being removed by aeration, as recommended by Sobel *et al.* (1945).

Uric acid was estimated colorimetrically by the method of Brown (1945), with slight modifications. The colour development appears to vary markedly with temperature and with the volume of the solution used for the estimation. The method finally adopted was therefore as follows. The sample was measured into a 25 ml. volumetric flask, and distilled water was added if necessary to obtain a final volume of 5 ml., 2 ml. each of the cyanide and urea solutions, followed by 1 ml. of the phosphotungstic acid reagent, were then added as described by Brown (1945). The solution was left in an incubator at 37° C. for 1 hr. and then made up to volume. The maximum colour development is thereby attained, and the colour remains stable for several hours. Readings were taken with a photoelectric colorimeter, using a red filter (Chance OR/2 at Plymouth and Ilford 608 at Cambridge). An approximately straight-line relationship was found to hold for values from 0·00 to 0·02 mg. uric acid, and care was taken to work within this range. The calibration curve was made using Folin's standard uric acid solution (1930).

Guanine and xanthine were precipitated with silver sulphate by the method of Gulland, Jordan & Threlfall (1947) and subsequently estimated colorimetrically with the phenol reagent of Folin (1927) as used by Hitchings (1941).

Allantoin was estimated by the method of Young & Conway (1942). Uric acid gives a similar colour reaction amounting to one-eighth of that obtained with an equivalent weight of allantoin. We therefore ignored the traces of colour which sometimes occurred with samples where uric acid was known to be present.

Results

The total soluble N.P.N. varied over a wide range in the course of the experiments. In general, the results of experiments yielding less than 1 mg. N.P.N. were discarded, since it was felt that partition data based on such small totals could not be relied upon in view of the experimental errors inherent in the analytical methods employed. In addition, we regard as unreliable all results obtained from experiments involving a high mortality, since in such cases a considerable proportion of the soluble N.P.N. is in all probability due to the disintegration and partial autolysis of dead organisms. It was found, for example, that a high percentage of casualties was often associated with exceptionally high values for amino-acid N. However, when dealing with large

populations, a few casualties are almost inevitable, but in most of the results which are presented here the deaths amounted to less than 1% and never more than 10% of the total.

The results are shown in Tables 2, 3 and 4, according to the respective habitats of the species. For comparative interest the wet weight of animals used in each experiment is recorded together with the total soluble N.P.N. obtained. In

Table 2. *Nitrogen partition in excreta of marine littoral and estuarine species*

Species	Exp. no.	Time (hr.)	Weight (g.)	Total N (mg.)	% NH_3-N	% Amino-acid N	% Urea-N	% Uric acid N	% Non-dialysable N	% Total N accounted
Gammarus locusta (A)	1	24	11	4·7	91	8	0	0	4	103
	2	41	11	9·3	74	5	3	0	2	84
	3	22	12	4·6	67	7	0	—	4	78
	4	24	11	6·8	87	7	0	—	3	97
	Av.	28	11	6·4	80	7	1	0	3	91
Marinogammarus marinus (A)	1	24	34	4·2	68	4	0	0	18	90
	2	28	19	2·0	81	4	0	—	0	85
	3	27	11	1·1	64	8	0	—	10	82
	4	21	11	1·4	75	4	0	—	4	83
	5	21	19	2·0	87	4	0	—	7	98
	Av.	24	19	2·1	75	5	0	0	8	88
Marinogammarus pirloti (A)	1	23	24	13·1	79	2	0	0	1	82
	2	23	30	11·7	92	1	0	—	3	96
	3	23	20	3·9	92	0	0	—	2	94
	4	23	18	3·2	86	4	0	—	2	92
	5	23	36	7·4	81	9	0	0	2	92
	6	24	24	8·6	90	0	0	—	3	93
	7	24	20	3·4	82	0	0	—	2	84
	8	24	18	4·1	96	0	0	—	2	98
	9	24	36	7·1	82	0	0	—	3	85
	Av.	23	25	6·9	87	2	0	0	2	91
Gammarus zaddachi (A)	1	26	11	4·1	88	2	4	—	4	98
	2	21	11	4·7	88	0	0	—	1	89
	3	21	8	5·0	73	6	0	—	4	83
	4	24	8	6·7	84	4	0	0	4	92
	Av.	23	10	5·1	83	3	1	0	3	91
Orchestia sp. (A)	1	22	34	6·5	51	26	3	0	7	87
	2	28	33	3·4	91	7	0	0	9	107
	3	25	30	5·5	62	12	0	—	6	80
	4	24	38	6·8	78	6	0	—	9	93
	5	21	38	6·6	66	6	0	—	11	83
	Av.	24	35	5·8	70	11	1	0	8	90
Ligia oceanica (I)	1	28	68	18·8	79	9	0	0	3	90
	2	24	91	9·2	58	13	0	—	6	77
	3	28	71	10·1	83	10	0	—	3	96
	4	25	30	4·8	89	5	0	—	3	97
	5	26	56	6·6	97	4	0	—	—	101
	6	23	54	3·1	73	3	0	—	—	76
	7	24	55	8·5	99	0	0	—	—	99
	Av.	25	61	8·7	83	6	0	0	4	91

addition the total non-dialysable N was estimated in most cases. This was done by dialysing a deproteinized aliquot for 24–48 hr. against running tap water and subsequent determination of the total N of the sac contents. This analysis was included since it seemed possible that a large proportion of the total N might be present in the form of products arising from incomplete digestion of the food proteins and partial autolysis of dead specimens. The results showed, however, that the percentage of non-dialysable N was usually relatively low, and a further investigation of the

nature of this fraction was therefore abandoned; there is in any case no reason at present to believe that this fraction contains compounds which could be regarded as true end-products of metabolism.

Guanine and allantoin were absent throughout and are therefore not included in the tables.

Marine littoral and estuarine species (Table 2). A very uniform type of nitrogenous excretion was revealed by these forms. About 90% of the total N.P.N. was accounted for in the six species studied, and the predominant nitrogenous constituent was ammonia in every case, the average values for each species ranging from 70 to 87% of the total N.P.N. The amino-acid N was variable but on the whole was lower in the aquatic than in the semi-terrestrial species, although even in the latter the values seldom exceeded 10%. Traces of urea were occasionally found, but it must be pointed out that the methods used for the estimations of urea and amino-acids are subject to rather large experimental errors; both depend on a relatively small titration over and above the sum of that due to volatile N in the sample and the

Table 3. *Nitrogen partition in excreta of terrestrial species*

Species	Exp. no.	Time (hr.)	Weight (g.)	Total N (mg.)	% NH_3-N	% Amino-acid N	% Urea-N	% Uric acid N	% Non-dialysable N	% Total N accounted for
Oniscus asellus (I)	1	24	38	1·8	44	12	0	5	14	75
	2	26	60	0·9	50	0	0	4	38	92
	Av.	25	49	1·4	47	6	0	5	26	84
Porcellio laevis (I)	1	26	50	2·0	58	2	0	2	10	72
	2	24	50	1·3	56	0	0	6	20	82
	Av.	25	50	1·7	57	1	0	4	15	77
Armadillidium vulgare (I)	1	24	50	2·0	59	0	0	4	—	63
	2	24	57	2·6	51	11	0	15	—	77
	3	26	58	2·1	55	7	2	6	—	70
	Av.	25	55	2·2	55	6	1	8	—	70

urease or ninhydrin blanks, and these are relatively large. We therefore feel justified in concluding that urea is at most a trivial end-product in these species.

Uric acid was present only in traces and never exceeded 0·1% of the total N.P.N.

Terrestrial species (Table 3). These proved the most difficult to handle, since the total amount of N.P.N. found in relation to the weight of animals used was always small. Roughly 50 g. woodlice were required to obtain 1–2 mg. total soluble N.P.N. Consequently it was possible to do only a few experiments, and these are not altogether conclusive. The N.P.N. recovered amounted to about 80% of the total, or 65% if the non-dialysable N is ignored. No obvious difference was observed in the behaviour of the three species studied. Roughly 50% of the N.P.N. was in the form of ammonia. Amino-acids were present in small concentrations only. Urea was absent throughout; the trace found in *Armadillidium vulgare* is within the limits of the probable experimental error. Some uric acid was always found, but this accounted for only 5–10% of the total N.P.N.

Fresh-water species (Table 4). The major excretory product was again found to be ammonia in both the fresh-water species studied, but an interesting deviation from

the other aquatic species described was discovered in this group. *Gammarus pulex*, which structurally is closely related to the marine and brackish *Gammarus* species, consistently produced small amounts of urea. The average value amounted to only 10% of the total N.P.N., but is almost certainly significant nevertheless. Uric acid, however, was entirely absent. In *Asellus aquaticus*, on the other hand, urea was never detected, but with this species some uric acid was always observed, although the mean of all experiments is only 5% of the total N.P.N. However, owing to the great sensitivity of the method for uric acid determination, this value is considerably more reliable than those which depend upon the steam distillation of ammonia.

Table 4. *Nitrogen partition in excreta of fresh-water species*

Species	Exp. no.	Time (hr.)	Weight (g.)	Total N (mg.)	% NH_3-N	% Amino-acid N	% Urea-N	% Uric acid N	% Non-dialysable N	% Total N accounted for
Gammarus pulex (A)	1	24	19	3·4	73	0	9	0	20	102
	2	25	18	5·1	69	0	6	0	13	88
	3	25	17	4·7	82	2	5	0	7	96
	4	25	19	5·8	66	2	6	0	10	84
	5	24	19	3·3	58	6	15	0	12	91
	6	24	20	4·1	71	10	10	0	7	98
	Av.	25	19	4·4	70	3	9	0	12	93
Asellus aquaticus (I)	1	24	9	3·6	66	4	0	9	—	79
	2	24	6	1·9	71	11	0	4	—	86
	3	24	11	2·1	55	3	0	5	—	63
	4	25	16	3·4	60	21	0	4	—	85
	5	23	16	2·9	56	10	0	1	13	80
	Av.	24	12	2·8	62	10	0	5	13	79

URIC ACID CONTENT OF WHOLE ANIMALS

Introduction

In view of the unexpected discovery that even in the terrestrial species the major excretory component is ammonia, it was decided to follow Needham's (1935) technique of extracting uric acid from whole animals, in order to discover whether a significant retention of uric acid occurs in any of the species studied. Such a retention might lead to false conclusions with regard to the nature of the main end-product of nitrogen metabolism.

Methods

The procedure adopted for the extraction of uric acid was as follows. Approximately 5 g. animals were ground up in a mortar with a little clean sand. Phosphate mixture (Benedict & Hitchcock, 1915) was then added and the whole thoroughly ground, transferred to a beaker, boiled gently for exactly 5 min. and then cooled rapidly. After centrifuging, the precipitate was once more ground with hot phosphate mixture, boiled for 3 min., cooled and centrifuged. The combined supernatants were diluted to 70 ml. 7 ml. of this extract were then made up to 10 ml. after the addition of 1 ml. each of the protein precipitants, resulting in a final concentration of 5 g. wet weight of tissue in 100 ml. After centrifuging off the precipitate, samples were taken for uric acid determinations. These proportions were adhered

to as far as possible, though in some cases greater dilutions were used as noted in the table.

Several experiments were carried out in order to check the reliability of this procedure:

(1) *Completeness of extraction.* The residues remaining after the extraction of 5 g. *Armadillidium vulgare* were ground up once more with hot phosphate mixture, boiled, cooled and centrifuged. The uric acid present in this second extract amounted to only 1% of that in the first.

(2) *Specificity of colour reaction.* Throughout the literature a general dissatisfaction is expressed with respect to the specificity of methods for the determination of uric acid which depend upon direct colour development in extracts of body fluids or tissues. It was therefore decided to investigate first of all the amount of residual colour remaining after incubation of the extract with urico-oxidase, prepared according to the method of Block & Geib (1947). This treatment resulted in complete removal of chromogenic substances in extracts of *Asellus aquaticus*, *Porcellio laevis* and *Oniscus asellus*. A small amount of residual colour was obtained with *Armadillidium vulgare*, but this was always less than 10% of the total chromogenic material and may have been due to incomplete oxidation of uric acid by the enzyme.

(3) *Recovery of uric acid.* Preliminary experiments were designed to investigate the effect of the presence of deproteinized tissue extracts on colour development with the phosphotungstic acid reagent. Thus 1 ml. of a deproteinized extract of *Oniscus asellus* gave a reading equivalent to 0·004 mg. uric acid. When 0·01 mg. uric acid was added, the reading obtained was equivalent to 0·014 mg. indicating that the intensity of colour had been neither enhanced nor inhibited appreciably. The average recovery from three such experiments using 1 ml. extract was 98%. With 2 ml. extract the average recovery in two experiments was 86%, indicating that some inhibition of colour development occurs if samples larger than 1 ml. are employed.

Finally, uric acid was added before the beginning of the extraction. Thus 5 g. *Gammarus pulex* were ground up in a mortar, and before continuing with the usual extraction procedure 1 ml. of Folin's standard solution (1930), containing 1 mg. uric acid, was added. Previous experiments had shown that the uric acid content of *G. pulex* amounts to 0·06 mg./g. wet weight of tissue, and the result was therefore calculated on the assumption that 100% recovery is equivalent to 1·3 mg. uric acid. In actual fact 1·2 mg. uric acid was extracted, giving a recovery of 92%. In a second experiment using *Oniscus asellus* the uric acid recovery amounted to only 79%. These results do, however, indicate that, in spite of its instability to heat, only a small proportion of uric acid is lost during the extraction procedure.

For the purposes of the present investigation the accuracy of the method seemed entirely sufficient, since it was desired merely to ascertain whether large differences of uric acid content exist between the various species.

Results

Table 5 shows that the results were remarkably uniform for the marine littoral and estuarine species, varying from 0·06 to 0·1 mg. uric acid/g. wet weight of tissue.

A similarly low uric acid content is found in one terrestrial species, *Oniscus asellus*, and in one fresh-water species, *Gammarus pulex*. On the other hand, *Armadillidium vulgare* contains approximately ten times as much uric acid as *Oniscus asellus*. The position of *Porcellio laevis* is a little uncertain, but on the whole it appears to conform with the group in which the uric acid content is low. However, the fresh-water hog slater, *Asellus aquaticus*, contains at least five times as much uric acid as *Armadillidium vulgare* and approximately one hundred times as much as its fresh-water associate, *Gammarus pulex*.

Table 5. *Uric acid content of whole animals*

Species	Habitat	Weight (g.)	No.	Approximate size	Final vol. extract (ml.)	Uric acid content mg./g. wet weight of tissue
Marinogammarus marinus (A)	ML	5·5	—	Random	100	0·07
		6·3	—	Random	100	0·07
Marinogammarus pirloti (A)	ML	3·6	—	Random	100	0·10
		2·7	—	Random	100	0·10
Orchestia sp. (A)	ST	5·1	—	Random	100	0·08
		5·5	—	Random	100	0·08
Ligia oceanica (I)	ST	5·1	—	Random	100	0·08
		5·2	—	Random	100	0·06
Oniscus asellus (I)	T	5·0	—	Random	100	0·08
		5·5	—	Random	100	0·09
		5·2	78	Random	100	0·09
Porcellio laevis (I)	T	5·0	—	Random	100	0·22
		5·1	83	Random	100	0·06
		5·0	70	Random	100	0·09
		2·5	17	Large (1·5–1·7 cm.)	50	0·13
Armadillidium vulgare (I)	T	5·1	—	Random	100	0·9
		2·7	45	Random	50	0·7
		4·9	—	Relatively small	100	0·7
		4·5	—	Relatively large	100	1·2
		0·8	182	Very small (0·3–0·5 cm.)	50	0·4
		1·1	7	Very large (1·3–1·5 cm.)	100	1·3
Gammarus pulex (A)	FW	5·4	—	Random	100	0·06
		5·2	—	Random	100	0·06
Asellus aquaticus (I)	FW	1·9	—	Random	40	6·5
		1·1	60	Random	100	8·1
		1·2	70	Random	100	6·7
		0·5	75	Small (max. length 0·7 cm.)	50	6·3
		0·5	18	Large (*c.* 1 cm.)	50	6·1
		0·5	19	Large (*c.* 1 cm.)	50	6·3

There appears to be no accumulation of uric acid with age in *Asellus aquaticus*, although more data are necessary in order to clarify this point. Some increase in uric acid content with size was, however, observed in *Armadillidium vulgare*, but when compared per unit body weight, the largest animals studied contained only three times the quantity extracted from the very smallest. The ratio for the average weights of the individuals in the two size groups is 1:36, which implies that the

protein turn-over involved in the building up of the body tissues of the larger animals must be very considerable. It would appear, then, that the amounts of uric acid retained in *A. vulgare* are relatively insignificant, and it cannot therefore be supposed that the nitrogen metabolism is essentially uricotelic even in this terrestrial species.

DISCUSSION

The six species studied at Plymouth were selected from a variety of habitats on the sea-shore and in estuaries, but, nevertheless, a very close similarity in the nitrogen excretion of these forms was evident. The nitrogen partition data leave no doubt that all are essentially ammonotelic. It therefore appears that even semi-terrestrial species, such as, for example, *Ligia oceanica*, which is frequently to be found in exposed and sunny places well above high-tide mark, have become adapted to this mode of life without concomitant modifications of the primitive ammonotelic type of metabolism.

Turning to the two fresh-water species we find that ammonia is again the major nitrogenous component of the excreta, but here either urea or uric acid occurs as a minor constituent. The amounts excreted (some 10% urea for *Gammarus pulex* and 5% uric acid for *Asellus aquaticus*) are relatively small, and it seems probable, therefore, that these components originate from the breakdown of purines rather than synthetically from the α-amino-N of proteins. If this were the case, it might be conceived that there exists among fresh-water organisms a tendency towards the loss of one or more of the enzymes involved in complete uricolysis, and that this modification has reached a more advanced stage in *A. aquaticus* than in *Gammarus pulex*.

The major excretory component of the woodlice proved also to be ammonia, amounting to some 50% of the total N.P.N. Considerably higher figures (70–90% NH_3-N) were obtained with the semi-terrestrial and aquatic species, but we believe that this difference is apparent rather than real and due in large part to the difference in the procedures adopted for the collection of the excreta. It seems highly probable that the more thorough extraction of the faecal pellets in the case of the terrestrial forms resulted in the appearance of a higher proportion of nitrogenous extractives derived from incompletely digested food materials. Thus it is perhaps significant that the non-dialysable fraction was markedly higher for the terrestrial species.

If, therefore, the nitrogen partition data of the excreta alone are considered, the terrestrial Isopoda must without doubt be regarded as ammonotelic, although small amounts of uric acid (5–10%) were present among the excreta. Now Needham (1935) has pointed out that uric acid excretion in certain snails is sporadic and occurs in large quantities at infrequent intervals. In such a case the analysis of the excreta, especially if these are collected over short periods of time only, might lead to misleading conclusions with regard to the identity of the major excretory component. However, even if such an erratic excretion of uric acid were typical also of the woodlice, it is very improbable that a major nitrogenous excretory product would be masked when large populations, rather than single individuals, are investigated in the course of a 24 hr. experiment, especially if, as in the present work, several such experiments

are performed. It must therefore be concluded that uric acid constitutes only a minor excretory product in terrestrial isopods and might again conceivably arise as the result of a loss of uricolytic enzymes, in particular of urico-oxidase.

A very striking difference was observed between the terrestrial species on the one hand and the semi-terrestrial and aquatic on the other, in the total amount of nitrogen excreted. Table 6 summarizes the mean values of mg. N.P.N./10 g./24 hr. excreted, for all experiments of each of the species studied. The variability of the results is considerable, but this is to be expected, since the level of nitrogen excretion must presumably depend largely upon the state of nutrition of the animals when collected, and some seasonal variation may also be involved. Table 6 does, however, show clearly that the total N.P.N. is very much smaller in the terrestrial than in any of the other species studied. It seems possible, therefore, that the woodlice have become adapted to a terrestrial mode of life, not by elaborating a non-toxic nitrogenous end-product, but rather by means of a general sparing or suppression of protein metabolism. Such a metabolic adaptation would be analogous to that found in the cleidoic egg (Needham, 1942).

Table 6. *Mean values for total N.P.N. excreted*

The figures in brackets denote the lowest and highest values respectively, which were obtained in individual experiments. For habitat abbreviations see Table 1.

Species	Habitat	No. of experiments	mg. N/10 g./24 hr.
Gammarus locusta (A)	ML	4	4·9 (4·2–6·2)
Marinogammarus marinus (A)	ML and E	5	1·1 (0·8–1·5)
Marinogammarus pirloti (A)	ML	9	2·9 (1·7–5·7)
Gammarus zaddachi (A)	E	4	6·0 (3·4–8·4)
Orchestia sp. (A)	ST	6	2·0 (0·8–3·4)
Ligia oceanica (I)	ST	9	1·3 (0·6–2·4)
Oniscus asellus (I)	T	2	0·3 (0·1–0·5)
Porcellio laevis (I)	T	2	0·3 (0·3–0·4)
Armadillidium vulgare (I)	T	3	0·4 (0·3–0·4)
Gammarus pulex (A)	FW	6	2·3 (1·7–2·9)
Asellus aquaticus (I)	FW	5	2·6 (1·9–2·9)

The relatively low level of nitrogen excretion in the terrestrial species might, however, be due to a partial retention of the nitrogenous end-products. It was for this reason that we investigated the uric acid contents of several of the species at our disposal. As expected, the marine littoral and estuarine species revealed a uniformly low uric acid content, ranging from 0·06 to 0·1 mg./g. wet weight of tissue, but when we turned to the terrestrial isopods interesting differences were observed. The uric acid content of *Oniscus asellus* falls within the low range obtained with the semi-terrestrial, marine and estuarine species. Whereas the values for *Porcellio laevis* are rather more variable, they are scarcely higher than those for *Oniscus asellus*, but *Armadillidium vulgare* contains about ten times as much uric acid as *Oniscus asellus*.

Considerations of morphological adaptations to aerial respiration indicate that *Oniscus* is the most primitive genus among terrestrial isopods, and there is no doubt that *Armadillidium* is the best adapted for a terrestrial existence. *Porcellio* occupies

an intermediate position, and it is conceivable that other species of this genus might have given rather higher uric acid values, for *P. laevis* itself frequents exceptionally damp habitats. Then again Waloff (1941) has demonstrated in a comparative study of the humidity reactions of *Oniscus asellus*, *Porcellio scaber* and *Armadillidium vulgare*, that the resistance to desiccation is greatest in *Armadillidium* and least in *Oniscus*.

An interesting correlation is therefore to be found between the degree of morphological and physiological adaptation to a terrestrial mode of life and the uric acid content of the body tissues. It seems more plausible, however, to attribute this increased uric acid retention in the more xerophilous species to a reduced rate of excretion, rather than to a fundamental difference in metabolism. Even for *Armadillidium vulgare*, the amounts of uric acid accumulated in the course of a lifetime are too small to suggest that ammonotelism has here been replaced by uricotelism. Our results indicate that the amount of uric acid accumulating in the course of one year is at most 0·3 mg. uric acid N/g. fresh weight of tissue. If we assume that the ammonia and uric acid excretion is roughly constant throughout the year, we can calculate that the total uric acid N excreted and stored in one year amounts to not more than one-fifth of the ammonia-N excreted during the same period of time.

If then a higher uric acid content is associated with more complete adaptation to terrestrial conditions, one would anticipate a uniformly low uric acid content in aquatic species in general. This is the case for the marine littoral and estuarine species as well as one of the two fresh-water species examined here, namely, the amphipod, *Gammarus pulex*. But in our other fresh-water form, the isopod, *Asellus aquaticus*, we found 5–7 times as much uric acid as in *Armadillidium vulgare*, and it is difficult to understand this extraordinary retention, in particular as there is apparently little or no increase with age. Indeed, it seems doubtful whether we are justified in the conclusion that uric acid in this species is derived solely from purines. It would therefore seem worth while to search for a uricogenic mechanism in *Asellus aquaticus*. If it is found that this species is capable of synthesizing uric acid *de novo*, it would then be desirable to investigate also the terrestrial Isopoda in order to decide whether these forms also possess a weak uricogenic system.

It is of interest to compare our data for the uric acid content of the tissues with those obtained by Needham (1935) using gastropods. Our values for *Armadillidium vulgare* are closely similar to those for some typically terrestrial pulmonates, as, for example, *Helix aspersa* and *Helicella itala*, although others, such as *H. virgata* and *Cochlicella acuta*, may contain three times as much and *Helix pomatia* even more than this. Furthermore, the uric acid content of *Asellus aquaticus* is greater than that of any of the gastropods studied by Needham (1935) with the exception of *Cyclostoma elegans* and possibly also *Helix pomatia*. It is known that *Cyclostoma* possesses 'concrementary glands' which contain dense deposits of uric acid (Meyer, 1925), and perhaps it is significant that large groups of cells filled with white 'urinary concrements' are characteristic also of *Asellus aquaticus* (Zenker, 1854; Němec, 1896). In both cases there appears to exist a specialized site for the storage of excretory substances which is separate from the excretory organs.

It seems, then, that our values for the uric acid content of isopods are similar to

Needham's (1935) data for gastropods, and it becomes evident that the whole problem of nitrogen metabolism in this latter group requires reinvestigation in order to decide whether Needham's assumption, that a uricotelic metabolism as indicated by a uric acid retention in the tissues, is in fact justified. In particular, it seems desirable to study the nitrogen partition of the excreta over long periods of time so as to determine whether ammonia is perhaps a major end-product of nitrogen metabolism even in some of the more xerophilous gastropods.

There is no doubt that uric acid is a metabolic end-product both in the terrestrial isopods and in *A. aquaticus*, but only future work can decide upon its origin. From the data here presented we incline to the view that the occurrence of uric acid in these isopods is due solely to a loss of the uricolytic enzymes, and that, in fact, all the species examined are essentially ammonotelic. We have not, however, eliminated the possibility that the terrestrial species might store some nitrogenous end-product other than uric acid, such as, for example, guanine. However, no trace of this was detected in the faecal pellets, and there exists no morphological evidence for the presence of any obvious organ of storage in the terrestrial Isopoda.

The embryonic development of the terrestrial Isopoda takes place inside a brood pouch, the latter being filled with fluid which is utilized to increase the volume of the developing young (Verhoeff, 1920). Ammonotelism in this group would then be in agreement with Needham's (1929) generalization: 'The main nitrogenous excretory product of an animal depends upon the conditions under which the embryos live, ammonia and urea being associated with aquatic prenatal life, and uric acid being associated with terrestrial prenatal life.' It is, however, very uncertain whether this generalization can in fact be applied to the invertebrates, since our knowledge with regard to the degree of cleidoicity in embryonic development is very deficient in most cases. The eggs of many insects, for example, possess specialized structures adapted for water absorption (Wigglesworth, 1939).

The occurrence of ammonotelism among semi-terrestrial and terrestrial species raises the problem of the degree of toxicity of ammonia in invertebrates. Sumner (1937) has shown that ammonia is indeed highly toxic for mammals and birds, the lethal level being reached with a blood concentration of 5 mg./100 ml., and Conway & Cooke (1939) came to the conclusion that ammonia, if present at all, does not exceed concentrations of 1 part in 10 million in the blood of the fowl, rabbit and man respectively. Similarly, Florkin (1943) found that the blood of certain poikilothermic vertebrates and also some insects (Florkin & Frappez, 1940) is almost free from detectable ammonia. This is not, however, the case for some molluscs and crustaceans, values ranging from 0·6 to 1·9 mg. NH_3-N/100 ml. having been observed in the snail, lobster and crayfish (Florkin & Renwart, 1939; Florkin & Frappez, 1940). Furthermore, according to Morgulis (1923), a single specimen of the prawn, *Panulirus argus*, can survive an injection of as much as 0·5 g. ammonium sulphate, and blow-fly larvae, as is well known, can tolerate exposures to relatively high concentrations of ammonia (Brown, 1938). It is clear that a more detailed study of the extent of the toxicity of ammonia towards invertebrates would be most valuable, and, in view of our results, determinations of the blood ammonia concentrations of *Ligia*

and the terrestrial Isopoda should be of interest. For it might be argued that the lower invertebrates are less susceptible to the toxic effects of ammonia than the more highly evolved insects and vertebrates, and that it is for this reason that ammonotelism is a possibility even among terrestrial representatives of the lower invertebrates.

SUMMARY

1. The nitrogen excretion of eleven species of amphipods and isopods, including marine, fresh-water and terrestrial forms, has been studied.

2. All species are essentially ammonotelic, since more than 50% of the total soluble N.P.N. of the excreta was present in the form of ammonia throughout.

3. The level of nitrogen excretion is appreciably lower in the terrestrial species than in any of the others, indicating that, in this group, adaptation to terrestrial conditions has been attended by a general suppression of nitrogen metabolism rather than by a transformation of ammonia to other, less toxic products.

4. Some 5–10% of the total soluble N.P.N. was present as urea in the case of the fresh-water amphipod, *Gammarus pulex*, and as uric acid in the terrestrial isopods as well as the fresh-water isopod, *Asellus aquaticus*. It is suggested that these minor excretory components might originate from purines as a result of the loss of one or more of the uricolytic enzymes.

5. In association with the excretion of uric acid some retention of this insoluble compound usually occurs, and it was found that among the terrestrial species the amount so stored parallels the degree of morphological and physiological adaptation to terrestrial conditions. The greatest accumulation of uric acid was, however, observed in the fresh-water species, *A. aquaticus*, and although such a storage cannot necessarily be taken as evidence for a partially uricogenic metabolism, this possibility must be borne in mind.

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REFINEMENTS IN POLARIZED LIGHT MICROSCOPY

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(With Plate 3 and Seven Text-figures)

INTRODUCTION

The general technique of polarized light microscopy in biology has been admirably described in the well-known article of W. J. Schmidt (1934). Other shorter accounts have been written, some of them in English, such as F. O. Schmitt (1947) and Hallimond (1949), but W. J. Schmidt's book (1934) remains the standard work for biologists. In one respect, however, this and all other accounts are lacking: they contain very little about the detection and measurement of very weak birefringence. No doubt this is largely because there has never been any particular call to detect such small retardations; the standard materials of the polarized light microscopist have always been such strongly birefringent objects as hair, nerve and muscle.

Various workers, however, have noticed that there is very weak birefringence to be found in many individual cells (see, for instance, Schmidt, 1937). Our own observations, particularly on mitotic figures and cell membranes (e.g. Hughes & Swann, 1948), soon showed that if this type of birefringence was to be treated quantitatively, it would be necessary, not only to develop suitable methods of measuring it, but also to increase the sensitivity of the microscope.

There are very few references in the literature concerning the least detectable retardation. Imbibition curves occasionally indicate minimum values of $\lambda/1000$ ($=5$ A.), and we have found in practice that this is about the limit of what can be measured with the mica plate compensator using the technique described by Schmidt (1934). The red blood cell ghost, for instance, was described by Schmitt, Bear & Ponder (1936) as having a very low retardation, presumably too weak to be measured; our own work has shown it to be about 4 A. We may then perhaps take 5 A. as the least that can be *measured*, for a typical biological object, using the standard methods. For the limit of detectability, W. J. Schmidt (1941) quotes 1·7 A., and Königsberger (1908, 1909) 0·55 A. Könisgsberger, however, was using large crystalline objects, and we can reasonably take 2 A. as the least that has been *detected* in typical biological material. Here and there are a few scattered references to the means that can be used to get maximum sensitivity. Bright light sources, small condenser apertures and strain-free lenses, for instance, are mentioned as being necessary. So far as we know, however, these references are nowhere collected, nor is there any critical examination of their relative importance. We have done no more than examine in greater detail the effect of these and a number of other fairly obvious

measures. The effect of using the whole range of simple improvements simultaneously is, nevertheless, considerable. The limit of detectability can be pushed from about 2·0 A. down to about 0·3 A. Using photographic methods as well, it is possible to measure down to the same limit. This is a startling figure, corresponding as it does to the retardation of a layer of well-oriented protein no more than one molecule thick.

IMPROVING THE SENSITIVITY OF THE POLARIZING MICROSCOPE

The sensitivity of a polarizing microscope can be increased in two ways: by increasing the contrast between the birefringent object and the rest of the field, and for visual observation by increasing the capacity of the eye to detect this contrast.* Neglecting for the time being the question of compensation, the contrast of an object under crossed 'polars'† depends on the strength of its birefringence, its position relative to the plane of the polarizer, and the brightness of the background. For a given object, lying with its optical axis in the focal plane and at 45° to the plane of the polarizer, the contrast can only be increased by lowering the background brightness. This brightness may be defined by what we shall call the 'extinction factor', which is the brightness of the field with polars parallel over the brightness with polars crossed. It can be shown that the least detectable retardation varies inversely as the square root of this 'extinction factor' (see equations 1 and 3, p. 231).

(1) *Increasing the extinction factor*

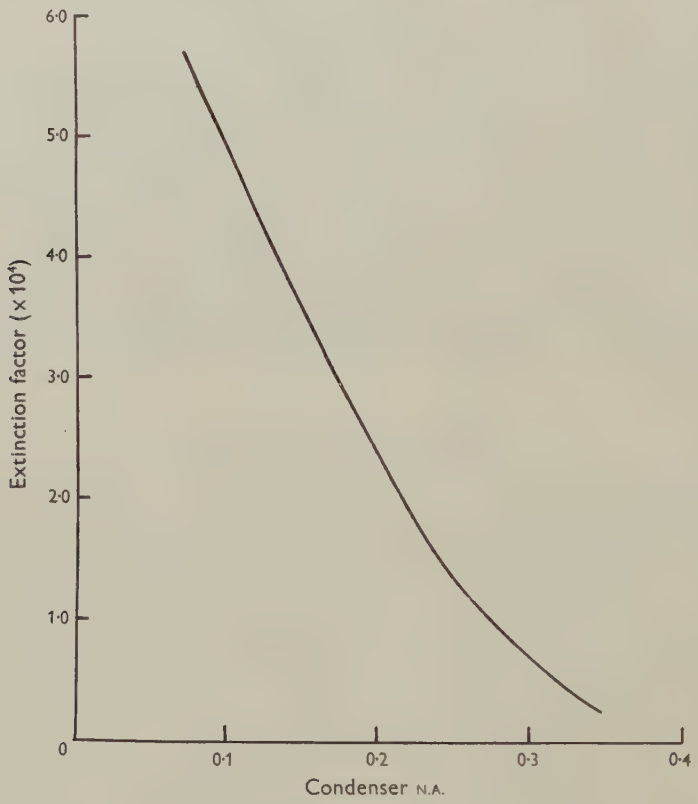
(*a*) *Polarizer and analyser.* The classical polarizers are, of course, Nicol or certain other similar prisms. At their best, they are extremely efficient, but they vary considerably. Hallimond (1944) gives a figure corresponding to an extinction factor of 20,000. We have measured, under a particular set of conditions, a pair of prisms from a Leitz CBMP polarizing microscope, and found a factor of 40,000; under the same conditions a pair of prisms from a Swift Survey microscope gave a factor of 7000. Recently, Polaroid sheets have been used instead of Nicol prisms. Hallimond (1944) measured an extinction factor of 60,000 for two mounted Polaroids which are now in the Geological Museum (No. M.I. 27964). Under our own standard conditions, however, most specimens we have tested have been far below this. We have, on the other hand, tested double thicknesses of Polaroid, and found, at best, a factor of about 45,000. Extinction factors depend to a large extent on the particular conditions of the test; using narrower apertures, for instance, we have obtained for Nicols and Polaroids factors of nearly 100,000. Our own figures, however, are not necessarily comparable with Hallimond's.

According to our own measurements, the best double Polaroids are slightly better than the best Nicols, but this is at the expense of a somewhat reduced light transmission. Parallel Nicols should transmit about 40% of light, but we have found in practice that, owing to correcting lenses and imperfections they transmit at the most

* Contrast is taken throughout as being: object brightness + background brightness/background brightness.

† Following the terminology of Hallimond (1949).

only 30%, and in some cases as little as 15%. The best double Polaroids in the parallel position transmit about 15%, and many of them transmit appreciably less. This loss of light is only a drawback for visual observation at high magnifications, and on balance there is probably not much to choose between the two systems. The essential is therefore that, whatever polarizers are used, they should be very good ones; but since, so far as we know, no makers ever advertise their extinction factors, there is no means of discovering whether they are good or bad, except by measuring them.



Text-fig. 1. Effect of condenser N.A. on extinction factor.

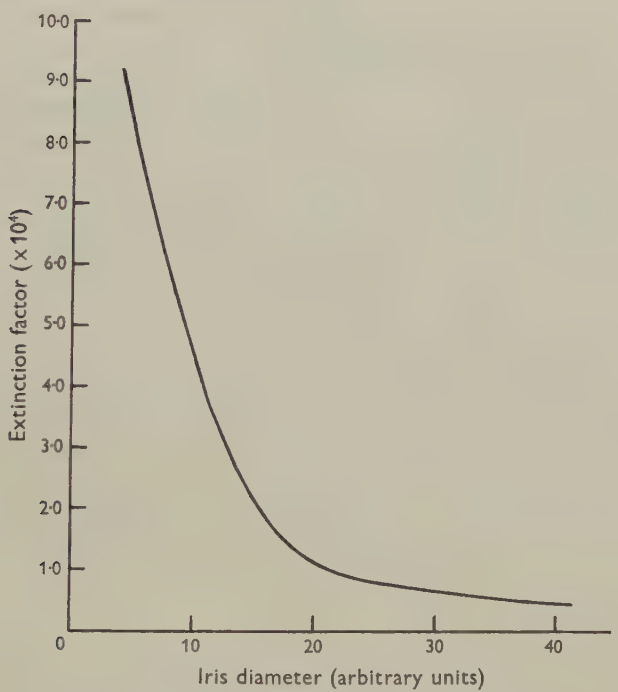
(*b*) *Rotation of the plane of polarization by the lens system.* When a beam of plane polarized light strikes an air-glass interface the plane of polarization of the reflected and refracted rays is rotated. This effect is negligible at small angles of incidence, but at the large angles to be found in wide aperture lens systems, it becomes serious and markedly reduces the extinction factor. The solution is to reduce the apertures throughout the system, to a point where the loss of light and loss of resolution can no longer be tolerated.

This can be done in two places: in the condenser and in the objective. In practice, the setting of the condenser iris is the more important, and its effect on the extinction factor is shown in Text-fig. 1. There is a steady increase of extinction factor as the

condenser aperture is reduced, but in fact the loss of light limits stopping down beyond a certain point by impairing the contrast sensitivity of the eye (see p. 232).

Objective aperture is best controlled, for a given magnification, by using low-power lenses and high-power eyepieces. For instance, we have often used a × 10 objective with a × 25 eyepiece, rather than, say, a × 40 objective with a × 6 eyepiece. Alternatively, objective aperture can be reduced by means of stops; we have a range of such stops to fit all objectives, and normally use the oil immersion 1·30 N.A. lens with one of them, cutting down its aperture to 1·0 N.A.

A simple means of eliminating the four glass surfaces with the greatest angles of incidence, is to oil above and below the slide. This makes little difference at low



Text-fig. 2. Effect of lamp iris on extinction factor.

apertures, but gives a considerable improvement with N.A.'s above about 0·7. In general, it is much better not to use high-aperture dry-lens systems, but rather high- or low-magnification oil-immersion systems.

(*c*) *Reflected light in the lens system.* Between the polarizer and the analyser there are some sixteen glass surfaces; at each of them a little light is reflected instead of being refracted. At normal incidence this amounts to about 4% at each surface; at greater angles rather more. This reflected light, as much as 50% of the total, will mostly go to and fro in the lens system, suffering a change of plane of polarization at every surface. Some of it inevitably finds its way into the field of view and spoils the extinction.

The amount of this reflected light is, of course, reduced by stopping down condenser and objective. It is also reduced by stopping down the lamp iris so that, under Köhler illumination, the object is surrounded in the field of view, by the minimum size of illuminated patch. As well as this, cutting down the lamp iris reduces the chance of the light beam striking uneven or faulty patches in the analyser. The effect of the lamp iris is shown in Text-fig. 2; it is surprisingly great.

Another way of reducing the amount of scattered light in lenses is to have them 'bloomed' with a thin film of the appropriate refractive index. 'Blooming' cuts down the reflexion at normal incidence from 4 to 1%, and increases the transmission accordingly. The increase in total transmission is itself valuable, while the reduced reflexion improves the extinction factor, particularly at wide apertures. For example, the extinction factor with a 0·65 N.A. objective is increased by 80% on blooming, and the total transmission increased by 15%.

Lenses vary greatly in the amount of light they reflect internally. Their performance in this respect can only be discovered by testing them, but the simpler and cheaper lenses are usually better than the more elaborate and expensive ones.

(*d*) *Strains in the lens system.* The need for strain-free objectives and condensers has been emphasized by Schmidt (1934). In fact, however, few if any lenses are perfectly strain free, and the need is rather for lenses with an even linear strain, the birefringence of which can be neutralized with a compensator. The same is, of course, true of slides and cover-slips. Uneven strain can be detected by the distortion it produces in the polarization cross in the back lens of the objective.

(*e*) *Scatter in the object.* Many birefringent objects scatter light because of granules they contain. Under these circumstances it is particularly desirable to stop down the objective in order to cut out as much as possible of the scattered light, without affecting the birefringence. Pl. 3, fig. 1, shows a sea-urchin egg in late anaphase photographed at a N.A. of 0·65. Pl. 3, fig. 2, shows a comparable stage photographed at 0·28 N.A. In the first, the birefringent aster is entirely obliterated by scattered light, while in the second, it is clearly visible. The loss of resolution at the reduced aperture is evident.

(*f*) *Cleanliness.* Great cleanliness is essential at every point when working with very small birefringences. A speck of dust on any of the surfaces in the 'magic circle' between the polarizer and the analyser may seriously reduce the extinction. Balsam crystals in the lenses and in permanent mounts have the same effect. Small crystals can be found in most immersion oils, and are liable to cause trouble; it is best to filter them off.

(2) *Increasing contrast by using a compensator*

So far we have not mentioned the use of a compensator to increase contrast. Under certain conditions, however, it may increase the sensitivity as much as four times, the equivalent of increasing the extinction factor sixteen times.

If a mica plate compensator is rotated so that its retardation adds to that of a birefringent object, the object becomes brighter more quickly than the background, until a maximum contrast is reached. Further rotation of the compensator causes the contrast to drop.

Provided that the retardations of the object and the compensator are small, and that the object is at 45° to the polarizer, the contrast is given by the equations below.*

If C = contrast without compensator,

C' = contrast with compensator,

f = extinction factor,

r = retardation of object in wave-lengths,

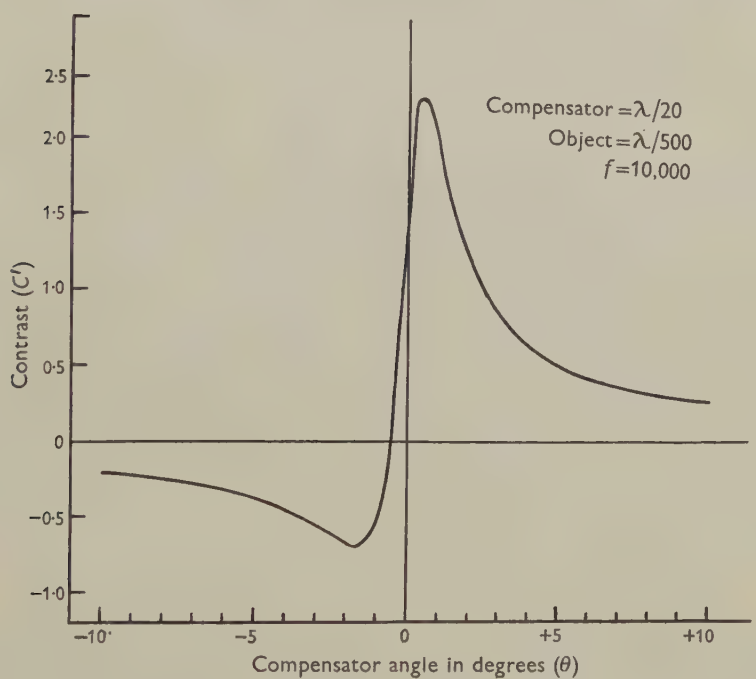
s = retardation of compensator in wave-lengths,

θ = angle of compensator from neutral position,

then

$$C = 4f\pi^2r^2, \tag{1}$$

$$C' = \frac{2rs \sin 2\theta + r^2}{s^2 \sin^2 2\theta + \frac{1}{4}f\pi^2}. \tag{2}$$



Text-fig. 3. Effect of compensation on contrast.

A curve of C' against θ is shown in Text-fig. 3 for $f = 10^4$, $r = 0{\cdot}002$ and $s = 0{\cdot}05$. The curve shows that the maximum contrast with a compensator is, in this case, 40% greater than without one. This maximum contrast is given by

$$C'_{(\text{max.})} = \frac{2}{\sqrt{\left(1 + \frac{1}{f\pi^2r^2}\right)} - 1}. \tag{3}$$

* So far as is known, these equations have not been given before. They can be derived from the general equation of Bear & Schmitt (1936) for the brightness of a birefringent object.

The ratio of $C'_{max.}/C$ rises in practice to a value of about 10 with a very weakly birefringent object. It should be noted that this ratio is independent of the retardation of the compensator. If the value of θ for maximum contrast is required, it can be found from the relation

$$\theta_{(C'_{max.})} = \frac{\sqrt{\left(r^2 + \frac{1}{f\pi^2}\right)} - r}{4s}. \qquad (4)$$

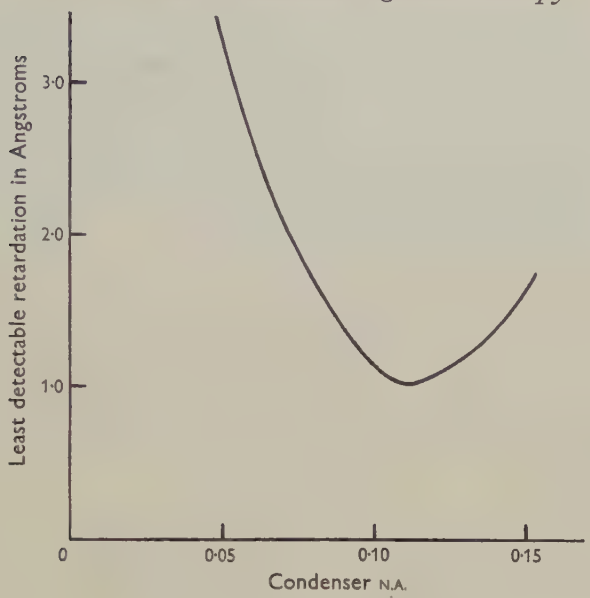
As well as increasing contrast and hence sensitivity, a compensator has three other uses. Two of these are well known: the measurement of sign and magnitude of birefringence, and the neutralization of birefringence due to strain in the optical system. The third, and less well-known use of a compensator, is to distinguish between brightness due to light scattering and brightness due to genuine birefringence. An object which is birefringent will darken when the compensator is moved in one direction, and lighten when it is moved in the other, while an object which is simply scattering will stay bright whatever the position of the compensator.

The type of compensator used is of some importance in getting a good extinction. It is usual to make small retardation compensators by opposing two plates of greater thickness because of the difficulty of cleaving very thin mica plates, but this procedure does not give such a good extinction as using a single sheet of mica. Cleaving mica to thicknesses of less than about $\lambda/10$ is not at all easy, but it can be done, and Messrs Cooke, Troughton and Simms Ltd. have cleaved small plates for us of about $\lambda/100$. As a simple home-made alternative to mica, 35 mm. cellulose acetate film base can be used. This is rather liable to contain flaws, but usually has a convenient retardation of about $\lambda/25$. Using standard conditions, we have found the following ratios of extinction factors: with a commercial double thickness compensator 1·00, with a cellulose acetate film base compensator 0·95, and with a single thickness mica plate compensator 1·67.

(3) *Increasing the contrast sensitivity of the eye*

It might be supposed that the greater the extinction factor the better, but in fact there comes a point when any increase in extinction is more than offset by a decrease in contrast sensitivity of the eye. This contrast sensitivity falls off rapidly at the low levels of illumination in the field of the polarizing microscope. Increased extinction necessarily means a reduced level of illumination, and may therefore result in a decreased sensitivity. In practice, however, it is not difficult to strike a balance between these two conflicting effects by slight adjustments to the condenser iris. This effect is shown in Text-fig. 4, where the maximum sensitivity is at a N.A. of 0·11.

There are two other ways of achieving a high level of illumination. The first is by using a strong light source. The approximate intrinsic brilliance of various possible light sources is given in Table 1.



Text-fig. 4. Effect of condenser N.A. on sensitivity.

Table 1. *Approximate values of intrinsic brilliance of various light sources, in candles/cm.*^{2}

High-intensity carbon arc	100,000
Low-intensity carbon arc	20,000
500 W. high-pressure mercury arc	20,000
250 W. high-pressure mercury arc	10,000
Pointolite	5,000
Tungsten filament	1000–2000

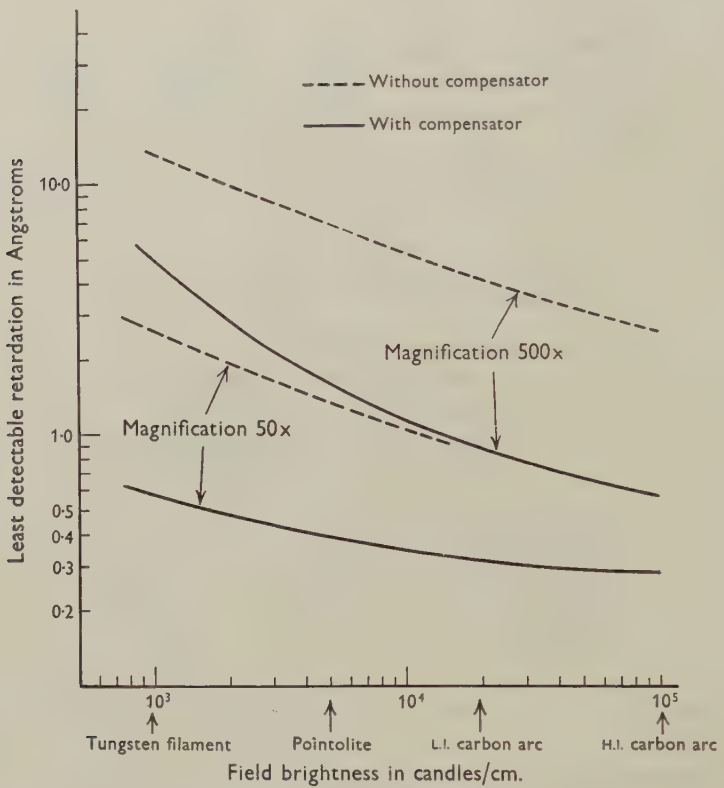
We have used a 500 W. mercury arc, as well as high- and low-intensity carbon arcs, and found them all satisfactory. The advantage that these sources give over a tungsten filament lamp or a Pointolite can be seen from Text-fig. 5. Strong light sources are also desirable when taking photographs. Though they do not, of course, increase the contrast sensitivity of the film, they do make it possible to use reasonably short exposures.

The second way of achieving a high level of illumination is by using a low magnification. The desirability of using low-power objectives has already been mentioned in connexion with reducing internal reflexion and eliminating change of plane of polarization; it is also desirable here, for halving the magnification will, of course, quadruple the level of illumination.

(4) *The result of applying all the improvements*

We have measured the sensitivity of our polarizing microscope, a Cooke, Troughton and Simms Research model, with the improvements mentioned above. A small fragment of mica, about 10μ in diameter was used as a test object, and the least

detectable retardation was measured by eye, using different light sources, with and without a compensator. The results are shown in Text-fig. 5. These curves show clearly the advantages of using a compensator and strong light sources. They show also that the limit of detectability for such an object is about 0·28 A. or 1/20,000th of the mean wave-length of white light. The limit with a more typical biological object is probably about 0·5 A.



Text-fig. 5. Effect of level of illumination on least detectable retardation.

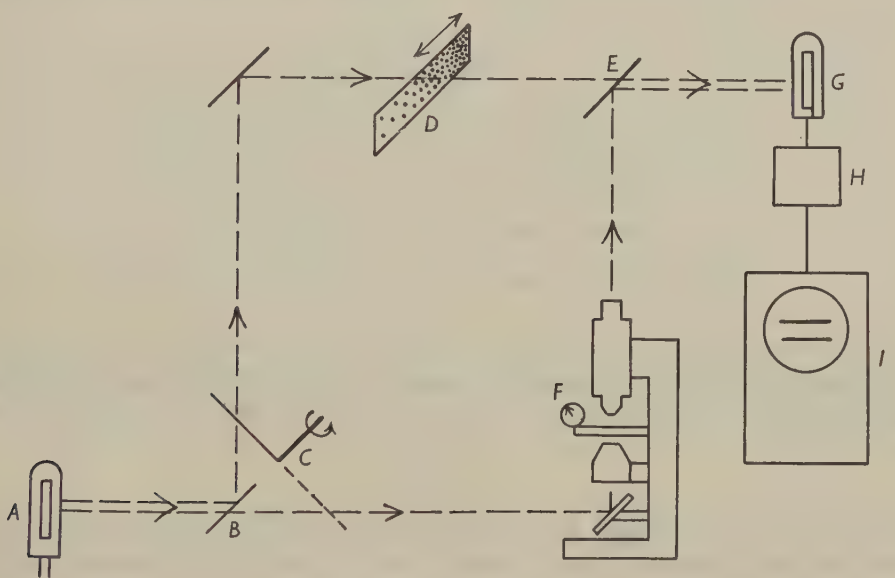
MEASURING EXTREMELY SMALL RETARDATIONS

The normal method of measuring small retardations, by using a compensator to obtain a position of maximum blackness of the object or equal intensity of the object and background, is not much use for the very small retardations in a cell. The objects themselves tend to be too small and dim to judge the positions with any accuracy, and they are usually not uniform, but varying rapidly in retardation from point to point. Furthermore, if the cell is living, the retardations may vary rapidly in time.

We have therefore developed a method for the photographic measurement of retardation, rather similar to the densitometric methods used in ultra-violet microscopy and elsewhere. We have usually taken the photographs on 16 or 35 mm. film, partly for convenience and partly because the levels of illumination are so low that

larger photographs would need impossibly long exposures. For the same reason we have nearly always used the fastest emulsions; either Kodak Super XX or Kodak R55, which is nearly as fast as Super XX and has a much higher contrast.

On the same strip of film as the photographs of the object to be measured, it is necessary to include a calibration. This is most easily done by taking a number of photographs of a blank field, with varying amounts of compensation. From such a set of negatives, a curve can be constructed relating the density of the film to retardation. Calibration can alternatively be based on the characteristic curve of the film and the retardation calculated, bearing in mind that the object brightness is proportional to the square of the object retardation. The characteristic curve of the film must, of course, be worked out for the particular conditions. When working out the retardation by either method it is essential to remember that photographs are normally taken with a compensator in position, and to allow for this.



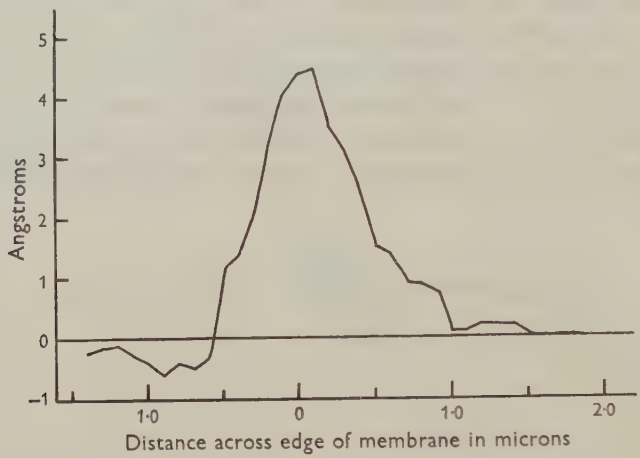
Text-fig. 6. Diagram of densitometer. *A*, ribbon filament lamp; *B*, beam-splitting cover-glass; *C*, rotating sector; *D*, optical wedge; *E*, beam-combining cover-glass; *F*, micrometer on microscope moving stage; *G*, photocell; *H*, cathode follower; *I*, amplifier and cathode-ray oscilloscope.

It remains only to describe the method of measuring film density. Since the negatives are small, and a considerable number of readings may be needed on a single one, we have developed an accurate densitometer working on patches of film down to 50μ square. It consists essentially of a microscope with a mechanical stage and dial gauge carrying the film to be measured, a logarithmic optical wedge, a battery-operated tungsten ribbon light source, a rotating sector plate letting the light alternately through the microscope and the wedge, and suitable lenses and mirrors for bringing the two rays of light on to a single photocell. The photocell is connected to a cathode follower and thence to the amplifiers of a cathode-ray oscilloscope.

Using a fast time-base two lines are produced, which merge when the two beams are exactly balanced. A diagram of the apparatus is given in Text-fig. 6.

It is possible to take several hundred readings an hour with this densitometer, to an accuracy which varies with the density of the film, but is of the order of 1%.

In this way retardations can be measured down to the limit of detectability by eye, or even lower. By way of illustration. Pl. 3, fig. 3, shows a human red blood cell ghost, with a maximum retardation of about 4 A. Text-fig. 7 shows an unsmoothed curve



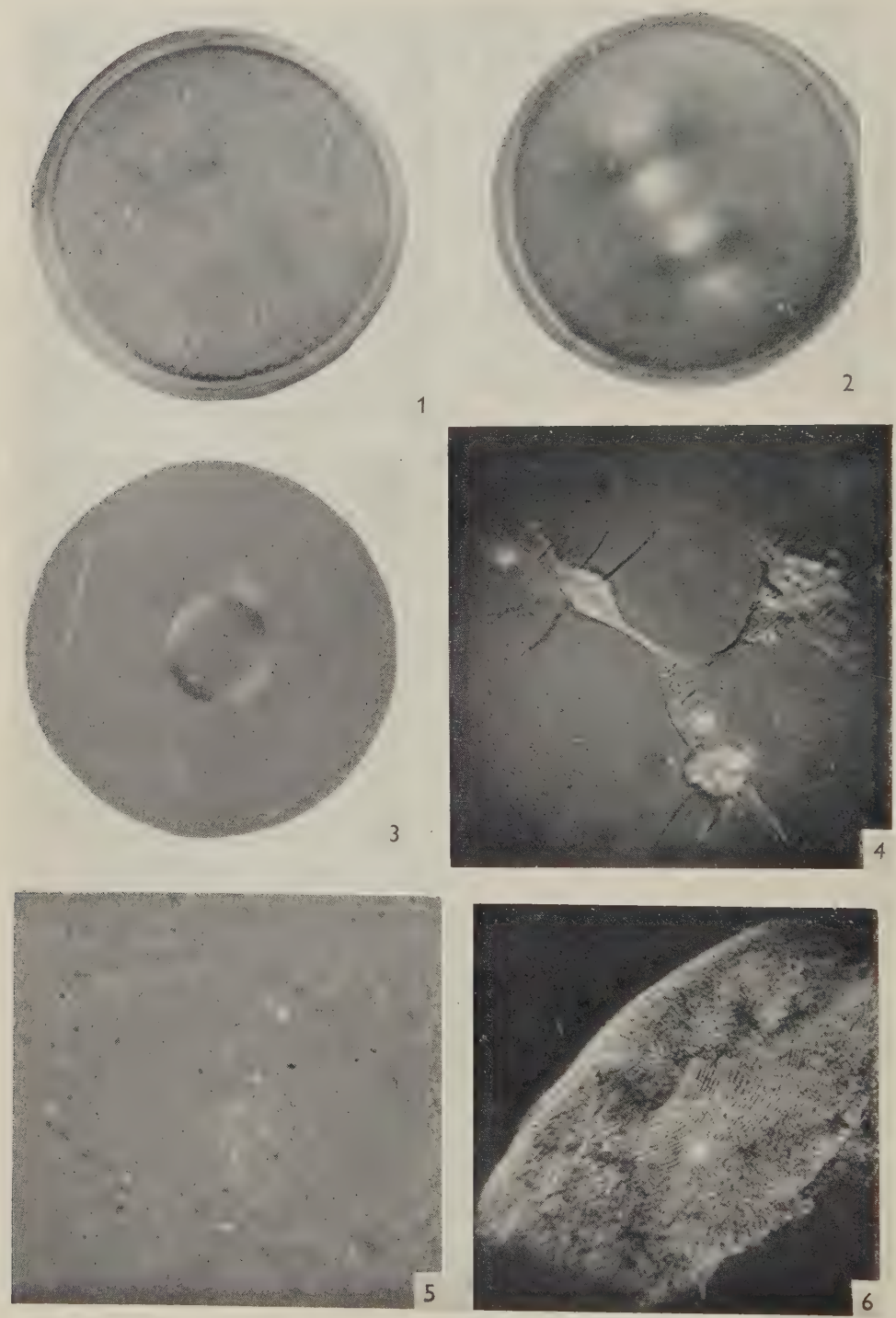
Text-fig. 7. Curve of retardation of the red blood cell membrane.

of retardation across the edge of the membrane. From the shape of such curves, it is possible to deduce a considerable amount of information about the thickness and structure of the membrane. Pl. 3, figs. 4–6, shows various other objects with low retardations.

SUMMARY

Various measures to increase the sensitivity of the polarizing microscope for visual observation are discussed. These include choice of polarizing apparatus, reducing condenser and objective aperture, reducing lamp iris, blooming lenses, use of mica plate compensators and use of bright light sources. By such means as these it is possible to detect retardations down to 0·28 A., or 1/20,000th of a wave-length. A photographic method for measuring retardations down to the same limit in small biological objects, is also described.

We are deeply indebted to Mr E. W. Taylor of Messrs Cooke, Troughton and Simms Ltd., for his great help in fitting our microscope with double Polaroids and a graduated compensator mount, as well as for blooming our lenses and making special low retardation mica plate compensators. We are also indebted to Dr L. E. R. Picken and Dr A. F. Hallimond for helpful suggestions at various stages of the work.



SWANN AND MITCHISON—REFINEMENTS IN POLARIZED LIGHT MICROSCOPY

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EXPLANATION OF PLATE 3

Fig. 1. Sea-urchin egg in late anaphase, photographed at 0·65 N.A. There is good resolution of granules, etc., but little or no sign of birefringence in the asters. × 500.
Fig. 2. Ditto, photographed at 0·28 N.A. There is poorer resolution, but the birefringence of the asters is clearly visible, as a result of cutting out scattered light. Maximum retardation about 5 A. × 500.
Fig. 3. Red blood cell ghost in glycerine. Maximum retardation about 4 A. × 2500.
Fig. 4. Culture of snail amoebocytes. Maximum retardation about 5 A. × 500.
Fig. 5. Emulsion of olive oil in sucrose solution with refractive indices of the two phases equal, stabilized with sodium hydroxide. The birefringence of the monomolecular layer of sodium stearate can be seen at the edge of the oil droplets. Maximum retardation < 2 A. × 350.
Fig. 6. Pellicle of *Paramecium.* Maximum retardation about 5 A. × 400.

AN ELECTRON DIFFRACTION STUDY OF THE CRYSTALLINE STRUCTURE OF THE LIPIDS IN THE PUPAL EXUVIAE OF *CALLIPHORA ERYTHROCEPHALA*

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(*Received* 14 *March* 1950)

(With Plates 4–6 and One Text-figure)

Electron diffraction techniques have been extensively used for the direct crystallographic examination of thin films of fatty materials, but they have hitherto not been applied to the study of natural membranes. These techniques provide more information than diffraction of X-rays about the atomic and molecular organization of surface structures. X-ray diffraction methods have been used successfully by Fraenkel & Rudall (1940, 1947) for elucidating the molecular organization of the chitin and protein components of the bulk cuticle framework in blowfly larvae; but the thin lipid-rich epicuticle constitutes a small proportion of the entire cuticle, and would not contribute effectively to the observed diffraction patterns.

Most membranes, including insect cuticle, are far too thick to permit penetration of the electron beam. The cuticle used as test material in the present paper is the thin membrane which covers the developing pupa of the blowfly larva, *Calliphora erythrocephala*. This pupal skin is relatively rich in waxy materials, which prevent excessive loss of water from the pupa in the same way as the waxy components exert a waterproofing action in more typical insect cuticles (Wigglesworth, 1945; Beament, 1945).

It is known that the waterproofing properties of the waxes of insect epicuticle are impaired at critical temperatures, which vary for different insects; and it has been suggested that each critical temperature corresponds to the transition point at which the molecules of crystalline wax become more mobile and assume a hexagonal type of packing, which is associated with an increase in intermolecular space (Beament, 1945).

It is the object of the present paper to characterize the structure of the lipids in the *Calliphora* pupal skin by comparison with artificial collodion membranes containing waxy materials of known crystalline structure; and to compare the changes in crystalline structure which occur when the membranes are exposed to temperatures at which the original crystalline organization breaks down.

MATERIAL AND METHODS

Collodion-wax membranes

The stock solution used was a 1% solution of collodion in amyl acetate; the fatty materials were dissolved in different samples of the stock solution in concentrations

of *c.* 3–5%. The best results were obtained when the solutions were almost saturated. The waxy materials consisted of *n*-fatty acids, unsaturated fatty acids, and *n*-paraffins.

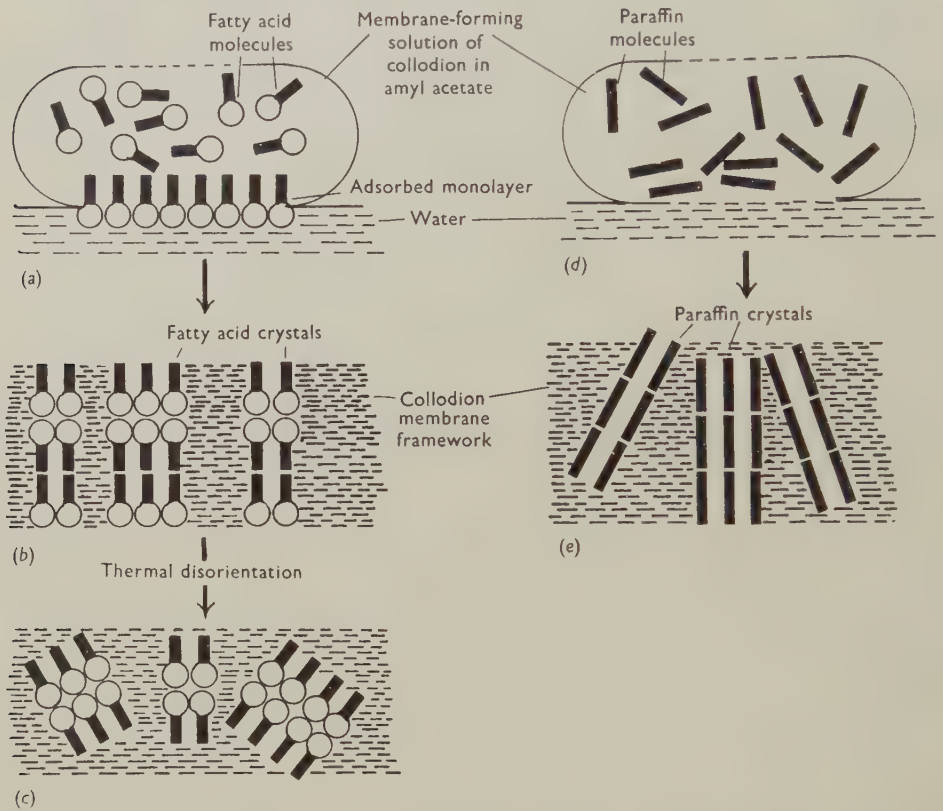
The technique used for the formation of the membranes was essentially similar to that which is in general use for the preparation of thin collodion supporting bases for the examination of specimens in the electron microscope. A drop of collodion solution spreads rapidly on a clean water surface to form a thin lens; after the solvent has evaporated a thin collodion membrane (*c.* 200 A. thick) is left floating on the surface.

If a long chain capillary active substance, such as a fatty acid, is present in the collodion solution, adsorption and orientation of a monolayer of fatty acid occurs at the amyl acetate-water interface during spreading and lens formation. Within the bulk phase of the lens, the fatty acid molecules are distributed at random, since there is no interface to attract the polar groups of the molecules (Text-fig. 1 *a*). As the solvent evaporates, crystallization of the fatty acid occurs within the lens. At the same time the collodion membrane is laid down and acts as a support for the fatty acid crystals.

When the waxy component in the membrane-forming solution is a long chain paraffin, no monolayer of paraffin molecules is formed at the amyl acetate-water interface (Text-fig. 1 *d*), for the molecules of paraffin are non-polar, but crystallization can occur in the bulk phase of the lens during membrane formation.

If there is a random orientation of fatty acid, or paraffin crystals in a collodion membrane, a relatively weak diffraction pattern will be obtained by transmission of the electron beam. The diffraction pattern will be more intense if the crystals show a common alinement of the molecular axes in relation to the basal plane of the membrane. The single layer of fatty acid molecules adsorbed at the amyl acetate-water interface during membrane formation will not contribute effectively to the diffraction of the electron beam, for the main scattering points are located in the thicker framework of the membrane. The purpose of comparing the diffraction patterns of collodion-fatty acid and collodion-paraffin membranes is to decide whether the orientation of the crystals in the bulk membrane frameworks is influenced by the original fatty acid monolayer. If the hydrocarbon chains of the molecules in the monolayer provide a basis on which the crystals in the membrane are deposited, it would be expected that a more intense pattern should be given by the collodion-fatty acid than by the collodion-paraffin membrane. This difference should disappear after the membranes have been exposed to high electron beam currents when the crystals melt or undergo transformation to a polymorphic crystalline modification. When the specimens are re-examined at the original beam currents, there will be no mobile fatty acid monolayer at the surface of the collodion-fatty acid membrane, and crystallization should occur at random in the collodion framework, as with the collodion-paraffin membrane (Text-fig. 1 *b*, *c*). If this takes place, there will be an irreversible fading of the diffraction patterns from the collodion-fatty acid membranes, which will be independent of any change in crystalline structure; but the original random orientation of paraffin crystals in the collodion membrane should give a

pattern which is substantially unchanged in intensity after melting and recrystallization has occurred (Text-fig. 1*e*).



Text-fig. 1. Stages n artificial membrane formation. (*a*) Lens formation by drop of collodion solution spread on water surface, showing adsorbed monolayer of fatty acid at amyl acetate-water interface, and random distribution of fatty acid molecules in bulk phase of lens. (*b*) Distribution of fatty acid crystals in supporting collodion membrane after evaporation of amyl acetate. The molecular axes of the crystals are normal to the basal plane of the membrane. (*c*) Random distribution of fatty acid crystals in membrane after thermal disorientation at high electron beam currents. (*d*) Lens formation by drop of collodion solution spread on water surface, showing random distribution of paraffin molecules in bulk phase of lens. (*e*) Random distribution of paraffin crystals in membrane after evaporation of amyl acetate.

Calliphora *pupal cuticle*

The thin pupal exuviae of *Calliphora* vary in thickness from 1·5 to 3·0 μ. Beament (1945) has calculated that the fat soluble waxes extracted by treatment in boiling chloroform can form a superficial layer constituting 5·8% of the thickness of the pupal skin. The skins were obtained from the puparia after the adults had emerged. The outer surface of each skin is hydrophobic, but the inner surface is hydrophilic. Portions of the membranes were floated on water with the hydrophilic surface in contact with the water. They were mounted on circular grids adapted for use with

the R.C.A. electron microscope and diffraction unit. A similar technique was used with the collodion-wax membranes.

The changes in diffraction patterns given by the pupal skins were observed after: (i) heating at high electron beam currents, (ii) extraction of the fat soluble waxes by chloroform, and (iii) disruption of the lipo-protein organization of the epicuticle by treatment in strong alkali.

Calculation of side spacings corresponding to Debye-Scherrer rings in diffraction patterns

Gold was volatilized *in vacuo* and deposited in a micro-crystalline film on to collodion bases mounted in grids. The crystals were of the face-centred cubic type ($a = 4{\cdot}07$ A.), and were orientated at random on the collodion bases. Each grid was inserted into the diffraction apparatus so that the gold film faced downwards. Photographs of the diffraction patterns were taken at constant voltage (50 kV.) across the electron gun, using a beam current of 200 μA. The side spacings represented by the Debye-Scherrer rings are inversely proportional to the ring diameters (Bragg's Law), the distance from the photographic plate to the specimen also remaining constant. For the four inner rings in a typical transmission pattern (Pl. 4, fig. 1), the side spacings may be calculated from the relationships in Table 1.

Table 1. *Calculation of spacing of planes from electron diffraction transmission pattern of micro-crystalline gold film deposited on collodion base. The crystals are of the face-centred cubic type* ($a = 4{\cdot}07$ A.)

Laue indices (hkl)	Relative ring diameters $\sqrt{(h^2+k^2+l^2)}$	Spacing of planes (A.) $a/\sqrt{(h^2+k^2+l^2)}$
111	$\sqrt{3}$	2·348
200	$\sqrt{4}$	2·035
220	$\sqrt{8}$	1·441
311	$\sqrt{11}$	1·225

In order to calculate the side spacings corresponding to the rings in the diffraction patterns from the collodion-wax membranes and insect cuticle, any particular ring in the gold diffraction pattern may be selected as a standard. The diameter of this ring is compared with those of the 'unknown' patterns, and the side spacings calculated in a manner similar to that used for the rings in the gold diffraction pattern.

It was observed that a slight distortion of the rings in the patterns sometimes occurred, owing to partial 'buckling' of the membranes on the grids. This introduced an experimental error in the measurement of ring diameters on the photographs, but was not of significance, since the crystalline structure of the fatty components could be characterized by relative ring diameters measured in the zones where the distortion was at a minimum.

RESULTS

Collodion-fatty acid membranes

Preliminary tests showed that the diffraction patterns faded rapidly at high beam currents (50 kV., 400 μA.) owing to excessive heating and melting of the waxy components in the electron beam. This fading was irreversible, but clearer patterns were obtained at lower beam currents (100–200 μA.) which could be photographed.

Myristic acid (C_{14}) gave a faint ring pattern, which faded rapidly even at low beam currents, but sharp patterns were obtained with the unsaturated elaidic (C_{18}) and brassidic (C_{22}) acids (Pl. 5, figs. 3, 5). With increase in chain length of the fatty acids, the diffraction patterns were more persistent to prolonged exposure to the electron beam. Octacosanoic acid (C_{28}) gave a pattern of continuous rings, but with the higher homologue, tetracontanoic acid (C_{34}) the rings were divided into discrete spots, showing that the membrane contained relatively larger and more isolated crystals (Pl. 5, figs. 1, 2).

Collodion-paraffin membranes

Somewhat irregular rings made up of isolated spots were apparent in the diffraction patterns of dotriacontane (C_{32}) and hexatriacontane (C_{36}) (Pl. 5, figs. 6, 7).

Crystalline structure

Brummage (1947) observed that the spacings given by thin films of the *n*-paraffins, tetracosane (C_{24}), triacontane (C_{30}), and tetratriacontane (C_{34}) agreed with the calculated spacings from an orthorhombic crystal structure with lattice constants $a = 7{\cdot}45$ A., $b = 4{\cdot}97$ A., $c =$ twice the length of the molecule (Müller, 1928). For crystals orientated in the (*hk*o) plane, the ring dimensions are given by the relationship

$$r = \lambda L \sqrt{\left(\frac{h^2}{a^2} + \frac{k^2}{b^2}\right)},$$

where r is the ring radius, λ the wave-length of the electron beam, L the distance between the specimen and the photographic plate, and h and k are Laue indices relating to the a and b axes, respectively. Since λ and L are constants, the ring radii are proportional to $\sqrt{[(h^2/a^2)+(k^2/b^2)]}$; the spacings represented by the rings can be calculated theoretically by substituting the appropriate values for h, k, a and b in the expression.

The *relative* ring dimensions in the diffraction patterns of the fatty acid and paraffin membranes are in agreement with the theoretical dimensions obtained from the above relationship; and the *absolute* dimensions, determined by comparison with the standard gold preparation, confirm the orthorhombic crystalline structure of the fatty materials in the collodion membranes. In Table 2, the theoretical values are based on an orthorhombic hydrocarbon chain lattice ($a = 7{\cdot}45$ A.; $b = 4{\cdot}97$ A.) adopted as a standard for comparison by Brummage (1947). The rings in the patterns

represent successive orders of diffraction by alternate carbon-carbon atom spacings of 2·54 A. in the zigzag hydrocarbon chains of the four molecules in the cross-section of the orthorhombic unit cell, when the molecular axes of the crystals are normal to the supporting membrane surface, but are otherwise distributed at random in the plane of the membrane.

Table 2. *Relationships between calculated spacings for an orthorhombic hydrocarbon chain lattice* ($a=7{\cdot}45$ A.; $b=4{\cdot}97$ A.) *and observed spacings for fatty acids and* n-*paraffins*

Laue indices (*hko*)	Side spacings (A.)						
	Calculated	Observed					
		Elaidic acid (C_{18})	Brassidic acid (C_{22})	*n*-acid (C_{28})	*n*-acid (C_{34})	*n*-par. (C_{32})	*n*-par. (C_{36})
100	7·45	7·41	—	—	—	—	—
010	4·97	4·95	—	—	—	—	—
110	4·13	4·15	4·15	4·12	4·14	4·12	4·10
200	3·73	3·65	3·68	3·71	3·68	3·70	3·72
210	2·98	2·94	2·96	—	2·94	—	—
020	2·48	2·44	2·47	2·45	2·43	2·42	2·46
120	2·36	2·31	2·34	—	—	—	—
310	2·22	2·18	2·20	2·20	2·20	2·20	2·22
220	2·06	2·03	2·04	2·03	2·01	2·00	2·02
320	1·76	1·73	1·74	—	—	—	—
230	1·51	1·49	1·50	—	—	—	—

Apart from differences in ring intensity and continuity, the ratios of the ring diameters correspond closely to those obtained by other workers for orthorhombic crystals of long chain compounds (Garrido & Hengstenberg, 1932; Natta & Rigamonti, 1935; Schoon, 1938; Coumoulos & Rideal, 1941).

Changes in crystalline structure after thermal disorientation

Irreversible changes in diffraction pattern after exposure of the specimens to high beam currents may be due either to a reduction in the degree of preferred orientation of the crystals in a particular plane relative to the incident electron beam, or to a change in crystalline structure. If the nature of the patterns remains unchanged, but there is a reduction in intensity, it may be concluded that there has been no polymorphic crystalline change, but only a reduction in the degree of preferred orientation, or alinement of the molecular axes of the crystals normal to the plane of the collodion membranes. On the other hand, partial fading of the patterns, together with the persistence of rings characteristic of other crystalline modifications, may indicate that apart from a change in preferred orientation, molecular reorganization of the molecules has occurred.

In the homologous series of aliphatic fatty acids, the melting-points become progressively higher with increase in chain length, and this introduced a difficulty in ensuring that all the specimens were sufficiently heated to permit changes in

crystalline structure to take place, especially with the fatty acids within the range of higher melting-points. Similarly, a certain amount of heating occurred even at the lower beam intensities (100–200 μA.) at which most of the diffraction patterns were photographed, and since the exposures varied from 5 to 10 sec., the patterns were examined on the fluorescent screen for 10–20 sec. before exposing the plates. For example, the pattern from myristic acid (C_{14}) faded so quickly that it was not possible to obtain a satisfactory photograph. When the plates were examined, only the diffuse ring pattern due to the collodion base itself could be seen. The sharp ring pattern given by elaidic acid (Pl. 5, fig. 3) could only be obtained by exposing the plate immediately after the specimen was inserted into the apparatus.

Preliminary tests showed that an exposure of 10 min. at 400 μA. was sufficient to melt or raise to the transition temperature the crystals of the longer chain derivatives; and this was adopted as a standard procedure in most cases. The specimens were first exposed at the lower beam currents, and photographs taken of the diffraction patterns. If these proved satisfactory, the beam current was raised to 400 μA. and maintained at this level for 10 min., after which the specimens were allowed to cool, and photographs of the diffraction patterns taken at the original beam intensity.

Saturated fatty acids. Pl. 6, fig. 1, shows the diffuse pattern given by the collodion base alone. After exposure to the high beam current, irreversible fading of the diffraction patterns of octacosanoic acid (C_{28}) and tetracontanoic acid (C_{34}) occurred, and only the inner ring corresponding to a side spacing of *c.* 4·12 A. remained in a diffuse form. But this ring showed hexagonal groups of spots or arcs in both patterns (Pl. 6, figs. 2, 3), suggesting that, apart from a reduction in the degree of preferred orientation of the crystals normal to the membrane surface, there was a change in crystalline structure from the orthorhombic to the hexagonal type of close packing in which the molecules are more mobile (Müller, 1932). Moreover, the change was irreversible, indicating that the original alinement of the *c* axes, or hydrocarbon chains of the crystals normal to the membrane surface was determined during the stage in membrane formation when the solubilized fatty acid was in contact with the adsorbed monolayer at the amyl acetate-water interface (Text-fig. 1, *a*).

Unsaturated fatty acids. The influence of thermal disorientation on the initial preferred orientation of the crystals is brought out clearly with elaidic acid (Pl. 6, fig. 4). Only a faint inner ring (*c.* 4·12 A.) persists, showing a hexagonal array of spots, similar to those in the patterns given by the saturated acids after thermal disorientation (Pl. 6, figs. 2, 3). In this connexion, the original pattern of elaidic acid (Pl. 5, fig. 3) is typical of the arrangement of orthorhombic crystals in which the *a* and *b* axes of the crystals are not orientated at random in the plane of the membrane, as with brassidic acid (Pl. 5, fig. 5), but are mainly alined in one direction in this plane. In the diffraction pattern, the six bright spots of the inner group are distributed so that four spots are on the (110) ring, and two on the (200) ring; moreover, the relative dimensions of the rectangular grid of spots on the (110) ring correspond exactly with those of the cross-section of the orthorhombic unit cell.

After thermal disorientation, this arrangement of the crystals changed, and

the molecules became arranged in the hexagonal type of close packing, in which there is a single common side spacing between the molecules. The hexagonal array of spots on the single ring indicates that there was an alinement of crystals normal to the membrane surface and also in the plane of the membrane (Pl. 6, fig. 4). A random distribution of hexagonal crystals in the plane of the membrane would give rise to a single continuous Debye-Scherrer ring (*c.* 4·12 A.) as in the pattern of the collodion-brassidic acid membrane after thermal disorientation at high beam intensity (Pl. 6, fig. 5).

Paraffins. Pl. 6, fig. 6 shows the change which occurred in the molecular organization of the crystals of hexatriacontane (C_{36}) after thermal disorientation and recrystallization. The original pattern consisted of isolated groups of small spots arranged in rings, the relative dimensions of which are consistent with an orthorhombic crystalline structure; the micro-crystals were distributed at random in the collodion framework, and the rings of spots were due to reflexion of the electron beam from planes in those crystals which were orientated perpendicular to the plane of the collodion supporting base (Pl. 5, fig. 7). The orthorhombic structure was maintained after the specimen was heated in the beam, but groups of bright larger spots appeared which could be resolved into three rectangular grids of spots on the (110) ring, each grid of which is associated with a single pair of spots on the (200) ring, representing an alinement of the *a* and *b* axes of the crystals in three directions in the plane of the membrane. Similar patterns have been described by Brummage (1947).

Calliphora *pupal skin*

The initial diffraction pattern of the *Calliphora* pupal skin was remarkably similar to that obtained with the fatty acids, especially with octacosanoic acid (Pl. 5, figs. 1, 4). After exposure to high beam currents, there was a partial fading of the ring pattern, and this change was irreversible, as with the artificial membranes (Pl. 6, figs. 2–5); but the persistence of a relatively intense ring corresponding to a spacing of *c.* 4·12 A. was similar to the change which occurred with brassidic acid (Pl. 6, figs. 5, 7), and suggested that a proportion of the thermo-labile lipids underwent a transition from an orthorhombic to a hexagonal lateral packing of the molecules.

The difference between the cuticle and the artificial membranes is seen in the persistence of an outer ring of *c.* 3·71 A. corresponding to the (200) spacing between the planes in orthorhombic crystals. Moreover, a rectangular grid of spots persisted on the inner (110) ring, and the appearance of a faint innermost ring (*c.* 4·92 A.) corresponded to the (010) spacing shown in the diffraction pattern of elaidic acid before thermal disorientation (Pl. 5, fig. 3). These changes are summarized in Table 3.

The main conclusion which may be drawn is that the thermo-labile lipids of the pupal skin are analogous to the fatty acid components in the artificial collodion membranes; but a proportion of the cuticle waxes, or lipids are relatively thermostable. The thermo-labile lipids undergo a crystalline transformation from an ortho-

rhombic to a hexagonal arrangement of close packing of the molecules, and this change is irreversible, as shown by the fading of the outer rings in the original diffraction pattern. The diffraction pattern of the thermo-labile lipids after thermal disorientation is superimposed on the pattern of the thermo-stable lipids, which retain the orthorhombic structure, and also show an alinement of the *a* and *b* axes of the crystals in the plane of the cuticle, which gives rise to the rectangular grid of spots on the (110) ring in the pattern.

Table 3. *Orthorhombic crystalline structure of lipids in* Calliphora *pupal skin*

Laue indices (*hko*)	Calculated spacings (A.)	Original spacings (before heating)	Spacings after heating
010	4·97	—	4·92
110	4·13	4·15	4·12
200	3·73	3·69	3·71
020	2·48	2·43	—
310	2·22	2·18	—
220	2·06	2·05	—

Distinction between fat soluble and bound lipids. Calliphora pupal skins were immersed in warm chloroform for 30 min., washed and mounted on grids in the usual way. It was assumed that this procedure resulted in the removal of the bulk of the thermo-labile, or fat soluble lipids. Pl. 6, fig. 8, shows that the residual lipids gave rise to a sharp ring pattern which could be related to the (110) and (200) spacings in the original pattern. The rings, however, were fainter, and a rectangular grid of spots on the (110) ring corresponded to a similar grid on the diffraction pattern after thermal disorientation. The pattern did not fade after the specimen was exposed to high beam currents, supporting the view that the thermo-labile lipids are fat soluble, whereas the thermo-stable lipids are 'bound' to the structural protein of the epicuticle layer in the pupal skin, and that this binding is responsible for the thermal stability.

Action of alkali on bound lipids. The bound lipids of the epicuticle are associated with the cuticulin layer, which is extremely resistant to the action of fat solvents, and cold concentrated mineral acids; but it is disrupted by hot alkali, or warm nitric acid saturated with potassium chlorate to liberate oily droplets (Kühnelt, 1928; Wigglesworth, 1947). The initial stages in the breakdown of the epicuticle framework were observed by immersing a pupal cast skin in 10% caustic potash for 15 min. The membrane was washed in distilled water and examined in the usual way. No ring pattern could be obtained, although the membrane was still intact. Similar negative results were obtained with preparations which had been treated first with chloroform to remove the fat soluble waxes. Clearly, the alkali destroys the organized structure of the epicuticle lipids before the membrane itself is broken down.

Electron microscope examination of pupal skin

Attempts to recognize a fine microscopic structure in the *Calliphora* pupal skin with the electron microscope proved unsuccessful. The cast skins were relatively trans-

parent to the electron beam, and the structure revealed at a screen magnification of 20,000 times was homogeneous (Pl. 4, fig. 2). This simplifies to some extent the interpretation of the electron diffraction patterns; the changes in pattern described were due to changes in molecular organization of the lipid components, and not to gross modifications in membrane structure.

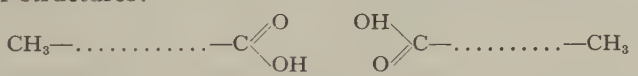
DISCUSSION

In comparing the artificial collodion-wax membranes with the *Calliphora* pupal skin, three aspects are of interest: (i) membrane formation, (ii) membrane structure, and (iii) membrane permeability.

Membrane formation

The membrane-forming material consists of a solution of nitrocellulose and fatty material in amyl acetate. Of these components, only the first two participate in the ultimate membrane. The nitrocellulose can be regarded as a fixed supporting framework which supports the crystals of waxy material after the solvent has evaporated, and in this respect the collodion fabric is analogous to the cuticulin layer of the epicuticle, which is associated with the fat soluble waterproofing waxes.

In the absence of nitrocellulose, a monolayer of fatty acid molecules would be formed at the amyl acetate-water interface, which could provide a starting-point for the deposition of crystals of fatty acid orientated with the molecular axes normal to the interface. Owing to the presence of the carboxyl group at the end of each molecule, association occurs between pairs of molecules, resulting in the formation of double-layer structures:



The intermolecular binding is ionic, and the lateral binding between the molecules in the double layers is dependent on the residual van der Waals' forces between the hydrocarbon chains.

The nitrocellulose macromolecule, however, can also form an orientated monolayer at the amyl acetate-water interface during membrane formation; and the non-polar side chains of the polymer monolayer could also offer points of attachment for the crystals of fatty acid which are deposited in the bulk membrane framework. There is a third possibility that both the fatty acid and nitrocellulose form a mixed monolayer at the solvent-water interface, so that crystal deposition could proceed by association with the hydrocarbon chains of the fatty acid monolayer, or with the non-polar side chains of the nitrocellulose monolayer.

There is a final possibility that the nitrocellulose membrane does not act simply as a physical or mechanical support for the wax crystals, but exerts a stabilizing influence on their orientation, which may involve polar and non-polar association with the side chains of the nitrocellulose in the bulk membrane framework.

In considering which of these factors is of major importance in the formation of a crystalline system in which there is a high degree of preferred orientation of the

molecular axes normal to the nitrocellulose membrane surface, the first clue is provided by a comparison between the diffraction patterns of the collodion-fatty acid and collodion-paraffin membranes. The orthorhombic crystalline structure is common to the waxy components in both systems, which shows that this is mainly determined by the lateral van der Waals' association between the hydrocarbon chains during crystallization. The rings are much more sharply defined and intense in the diffraction transmission patterns of the collodion-fatty acid than of the collodion-paraffin membranes, and this is consistent with surface active nature of the fatty acid molecules, and with the non-polar nature of the paraffin molecules. The latter do not become orientated to form a monolayer at the solvent-water interface during membrane formation, and the ring patterns of isolated pairs of spots are those which would be expected from a random distribution of micro-crystals in which there is no common axis of alinement normal to the membrane surface.

Since a common feature of the formation of the collodion-fatty acid and the collodion-paraffin membranes is the deposition of the collodion framework, van der Waals' association between the fatty components and the non-polar side chains of the collodion fabric cannot be the main factor which influences the preferred orientation of the fatty acid crystals in the membrane, for this factor would also operate with the paraffin crystals. This leaves the possibilities that the orientation of the fatty acid crystals is determined by polar association with the polar side chains in the bulk collodion framework, or by non-polar association with the monolayers of collodion and fatty acid formed at the amyl acetate-water interface. The initial collodion monolayer can be regarded as an integral part of the fixed framework of the system, for it is incorporated in the ultimate membrane which is formed.

The irreversible fading of the diffraction patterns of the collodion-fatty acid membranes when these are exposed to high electron beam currents provides a further clue which restricts these possibilities to one in which the limiting factor is the mobile monolayer of fatty acid formed at the solvent-water interface. After thermal disorientation, the fatty acid molecules can still associate with the fixed collodion framework, but a solvent-water interface is no longer present, as the original adsorbed fatty acid monolayer is now incorporated in the bulk crystalline phase and is subject to similar thermal disorientation. This fatty acid monolayer is therefore a limiting factor in determining the common orientation of the molecular axes of the fatty acid crystals which are deposited in the collodion framework, and this acts mainly as a mechanical support for the three-dimensional crystalline system.

Membrane structure

The diffraction pattern of the collodion-C_{28} fatty acid membrane (Pl. 5, fig. 1) corresponds closely to that of the *Calliphora* pupal skin (Pl. 5, fig. 4). This observation is of particular interest in view of the conclusion reached by Beament (1945) that the fat soluble waxes extracted by chloroform from *Calliphora* pupal exuviae are sufficient to form a layer $0{\cdot}18\,\mu$ thick on the surface of the epicuticle. The artificial collodion membranes were only about 150–200 A. thick. Adopting Bergmann's (1938) view as a basis for comparison that the average chain length of

the fat soluble epicuticular waxes from silkworm exuviae is about C_{30}, a layer of wax 0·18 μ thick would correspond roughly to forty single molecular layers, assuming that the hydrocarbon chains are orientated perpendicular to the surface of the layer, and that the vertical distance between two adjacent carbon atoms on the zigzag chains is about 1·43 A.

Since forty molecular layers of wax cannot be accommodated within the collodion framework, it is likely that the micro-crystals project to a considerable degree above the surface of the dry collodion base to form a superficial layer of wax which is continuous with the underlying waxy components in the supporting collodion membrane. This is to be expected, for crystallization proceeds during membrane formation before the solvent has evaporated, and crystal growth is influenced by the original minute nuclei in the membrane-forming solution.

After the *Calliphora* pupal skin has been treated with chloroform, it still gives a well-defined diffraction pattern, which is thermo-stable. This introduces an experimental difficulty in correlating the artificial with the natural membranes, for chloroform may displace both the superficial waxes from the epicuticle surface, and also the waxes which impregnate the underlying cuticulin layer. The waterproofing waxes are secreted after the cuticulin layer is formed (Wigglesworth, 1945, 1947, 1948), and the essential condition for the formation of a superficial layer of crystals in which the hydrocarbon chains are perpendicular to the cuticulin surface is that the waxes should be mobile and in contact with a water surface. This condition would be fulfilled if the wax is solubilized by a more hydrophilic lipid, such as a phospholipid, as suggested by Beament (1945), during the process of secretion. A droplet of solubilized wax on the hydrated surface of the cuticulin layer would be analogous to a lens of a solution of wax in amyl acetate spread on a water surface (Text-fig. 1 *a*), ignoring for the moment the presence of collodion. If the wax droplets spread or coalesce on the cuticulin surface an orientated monolayer of wax would be formed which could initiate the alinement of crystals in the superficial layer, although it is necessary to postulate that some change occurs in the solubilizing agent which leads to this crystallization. Here also, as with the artificial systems, crystals could extend inwards into the cuticulin framework, for this layer must necessarily be permeable to the solubilized wax during the process of secretion—a conclusion which is consistent with the absence of holes or 'pore canals' in the *Calliphora* pupal skin (Pl. 4, fig. 2).

While the electron diffraction evidence does not show whether the thermo-labile lipids of the pupal skin are entirely distributed on the surface or impregnate partially the underlying layer of the epicuticle, the difference in thermal stability between the fat soluble and 'bound' lipids suggests that the latter are linked in some way with the protein components of the epicuticle, although it is also possible that the distinction between the 'free' and 'bound' lipids is one of degree. The persistence of the orthorhombic crystalline structure of the bound lipids can be detected after the transition of the free lipids from the orthorhombic to the hexagonal structure when the pupal skin is exposed to high temperatures in the electron beam; and also after the free lipids are removed by chloroform. The presence of a rectangular grid of

spots on the (110) ring after such treatments suggests that there is an alinement of the *a* and *b* axes of the crystals of bound lipids in the plane of the epicuticle, and this may reflect a similar alinement of the protein fibrils in the epicuticle framework.

Membrane permeability

From the point of view of membrane permeability, the results are consistent with the findings of Wigglesworth (1945) that when insects are exposed for some time to high temperatures, the waterproofing properties of the waxes in the epicuticle are permanently impaired. The present results offer an explanation involving a crystalline transition from an orthorhombic to a hexagonal type of close packing. While this change is reversible with the long-chain paraffins (Müller, 1932), the long-chain fatty acids used in the formation of collodion-wax membranes in the present work are more typical of insect waxes, and the transformation in these systems, and in the *Calliphora* pupal skin is irreversible under the particular experimental conditions. As suggested by Beament (1945), such a change involves an increase in intermolecular space between the hydrocarbon chains of the crystalline wax molecules, and this would increase the permeability of the wax system to water. Müller (1932) has correlated the change from an orthorhombic to a hexagonal crystalline lattice with the anisotropic thermal expansion of the orthorhombic lattice in which the molecules behave as freely rotating cylinders before melting occurs. The expansion is greater along the *a* axis of the unit cell than along the *b* axis; the thermal expansion is much smaller along the *c* axis, owing to the strong binding force between the carbon atoms in the chains.

SUMMARY

A comparison is made between the known crystalline structures of long-chain fatty acids, paraffins, and the free and bound lipids in the epicuticle of *Calliphora* pupal exuviae.

Artificial collodion-wax membranes give electron diffraction patterns which are similar to those of the insect cuticle, indicating that the free, or thermo-labile waxes in the epicuticle consist of a three-dimensional arrangement of orthorhombic micro-crystals oriented perpendicular to the cuticle surface, but otherwise distributed at random.

The thermo-labile waxes of the epicuticle undergo irreversible disorientation when the pupal skin is exposed to high beam currents, and similar changes occur with the artificial collodion-fatty acid membranes. This is correlated with the mechanism of membrane formation from a solution of fatty acid and collodion in amyl acetate. When a drop of solution is allowed to spread on a water surface, a lens is formed and a monolayer of fatty acid adsorbed at the amyl acetate-water interface. This monolayer acts as a basis on which an alinement of crystals occurs in the bulk framework of the lens as the solvent evaporates. The crystals are immobilized in the membrane of collodion which is simultaneously deposited. When the dry membrane is heated no monolayer is present to act as an organizing factor in crystal

growth, which occurs at random in the bulk framework of the collodion membrane. Similar random crystallization occurs in collodion-paraffin membranes, where the waxy component is non-polar, and does not form a monolayer at the solvent-water interface during membrane formation.

Polymorphic crystalline modifications are observed after the collodion-fatty acid membranes and the *Calliphora* pupal skin are exposed to a more intense beam. Apart from a reduction in the degree of preferred orientation of the thermo-labile waxy components, there is a transition from an orthorhombic to a hexagonal type of lateral close crystalline packing. This is correlated with the irreversible increase in permeability of the cuticle to water at high temperatures.

After the thermo-labile lipids are removed from the pupal skin by chloroform, the residual lipids give a diffraction pattern which does not fade when the cuticle is heated. The pattern suggests that the bound lipids can form orthorhombic crystalline aggregates in the cuticulin layer, and that the thermo-stability is due to association between the lipids and the structural proteins of this layer.

The organized structure of the bound lipids is destroyed when the pupal skin is in contact with strong alkali before the membrane itself is disrupted.

The pupal skin is homogeneous when examined with the electron microscope at a screen magnification of 20,000 times.

The structural relationships between the free and bound lipids of the epicuticle are discussed.

The electron diffraction diagrams were taken with the R.C.A. electron microscope and diffraction unit in the Cavendish Laboratory, Cambridge, by kind permission of Dr V. E. Cosslett. I am indebted to Mr R. G. Allen and Mr J. W. Menter for helpful advice and discussions; and to Mr G. R. Crowe for technical assistance. The samples of pure chemicals used were kindly supplied by Dr J. H. Schulman.

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EXPLANATION OF PLATES

PLATE 4

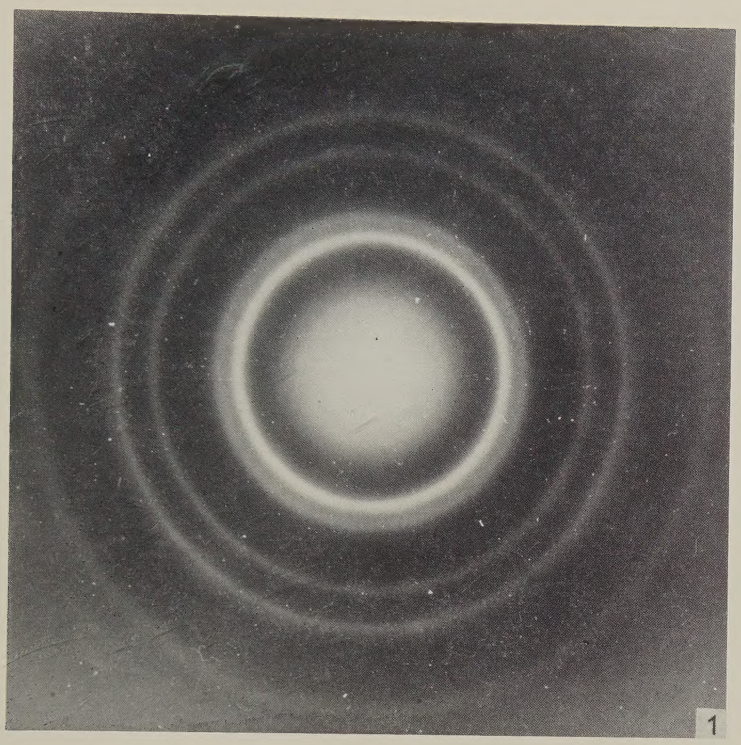
Fig. 1. Electron diffraction transmission pattern of micro-crystalline gold film deposited on collodion base. Table 1 refers to the side-spacings represented by the four inner rings.
Fig. 2. Electron micrograph of *Calliphora* pupal skin, showing homogeneous structure.

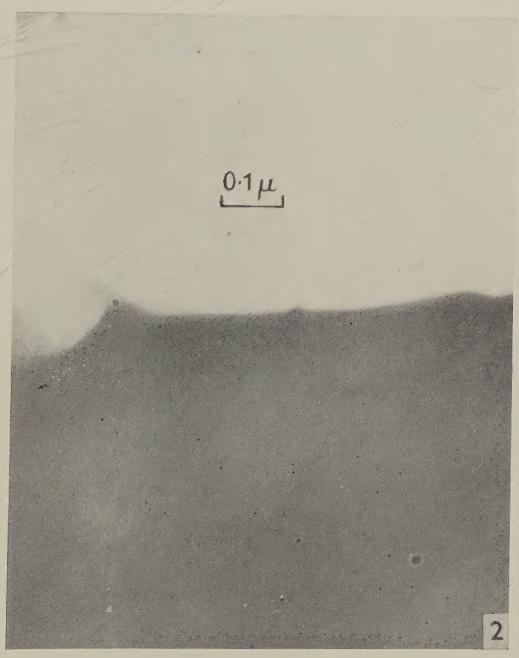
PLATE 5

Electron diffraction transmission patterns given by collodion-fatty acid, collodion-paraffin membranes, and by *Calliphora* pupal skin before exposure to high electron beam currents.
Fig. 1. Octacosanoic acid (C_{28}).
Fig. 2. Tetracontanoic acid (C_{34}).
Fig. 3. Elaidic acid (*trans*) (C_{18}).
Fig. 4. *Calliphora* pupal skin.
Fig. 5. Brassidic acid (*trans*) (C_{22}).
Fig. 6. Dotriacontane (C_{32}).
Fig. 7. Hexatriacontane (C_{36}).

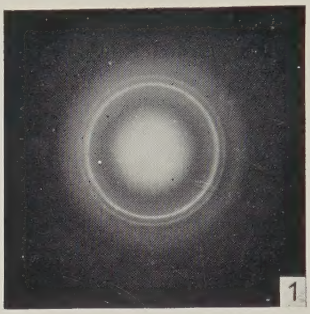
PLATE 6

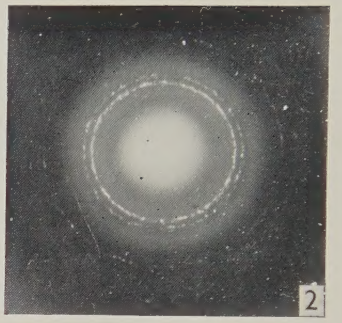
Electron diffraction transmission patterns of collodion, collodion-fatty acid, collodion-paraffin membranes, and of *Calliphora* pupal skin after thermal disorientation at high beam intensity.
Fig. 1. Collodion. The diffuse Debye-Scherrer ring is due to the collodion base alone.
Fig. 2. Octacosanoic acid (C_{28}). There is a bright hexagonal array of diffuse spots and a weaker hexagonal pattern, representing two groups of crystals covered by the incident electron beam. In both the molecules are oriented perpendicular to the membrane surface, but the groups are not alined in the plane of the membrane.
Fig. 3. Tetracontanoic acid (C_{34}). The pattern is weak, but essentially similar to that in fig. 2. Two groups of hexagonal crystals have been covered by the electron beam.
Fig. 4. Elaidic acid (C_{18}). A single weak hexagonal pattern can be recognized.
Fig. 5. Brassidic acid (C_{22}). The continuous Debye-Scherrer ring represents a random distribution of hexagonal micro-crystalline aggregates in the plane of the membrane.
Fig. 6. Hexatriacontane (C_{36}). The spots are distributed in two rings corresponding to the arrangement shown in the elaidic acid pattern (Pl. 5, fig. 3). The orthorhombic crystalline structure is maintained after melting and recrystallization. The electron beam has covered three groups of crystals. The rectangular grids of spots on the (110) ring can be identified from the corresponding pairs of bright spots on the (200) ring, showing that the crystals are alined in three directions in the plane of the membrane.
Fig. 7. *Calliphora* pupal skin. The most intense ring is due to the presence of a random distribution of hexagonal crystals of thermo-labile lipids similar to those responsible for the diffraction pattern of brassidic acid (fig. 5). The inner and outer rings are due to the thermo-stable bound lipids.
Fig. 8. *Calliphora* pupal skin after treatment with chloroform. The diffraction pattern is due to the bound lipids. The (110) and (200) rings have sharpened. The rectangular grid of spots on the (110) ring corresponds to that in fig. 7.

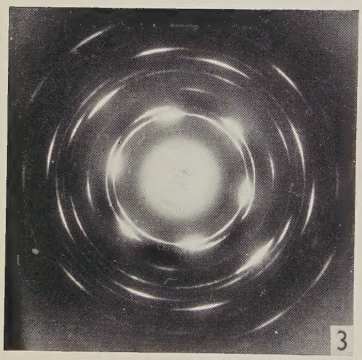


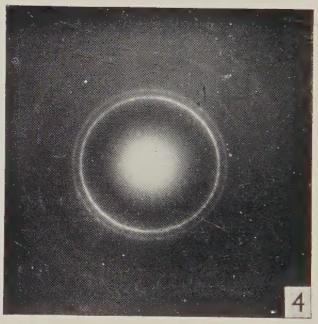


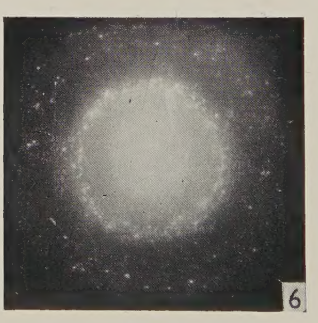
HURST—STUDY OF CRYSTALLINE STRUCTURE OF LIPIDS

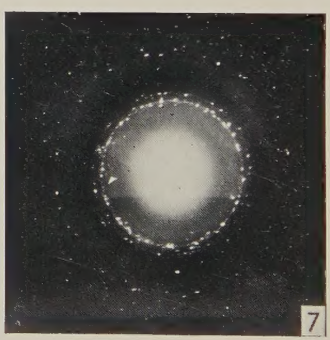












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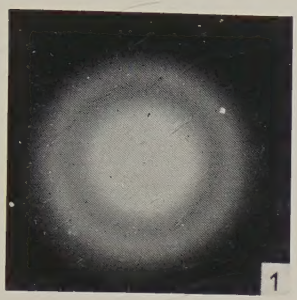

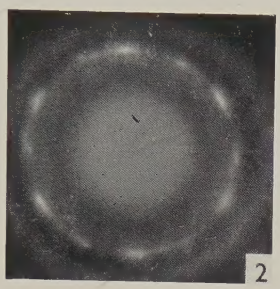

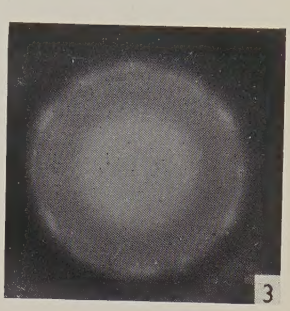

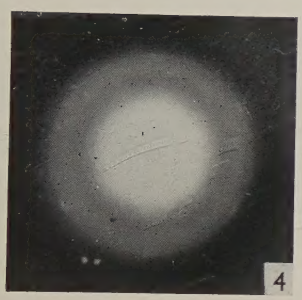

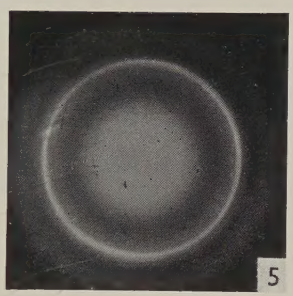

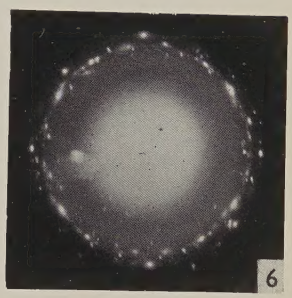

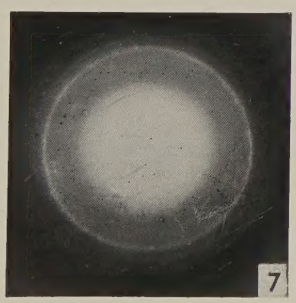

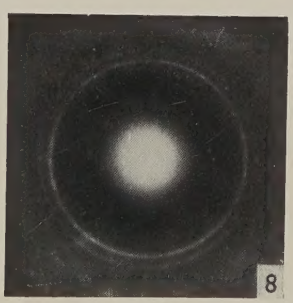

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